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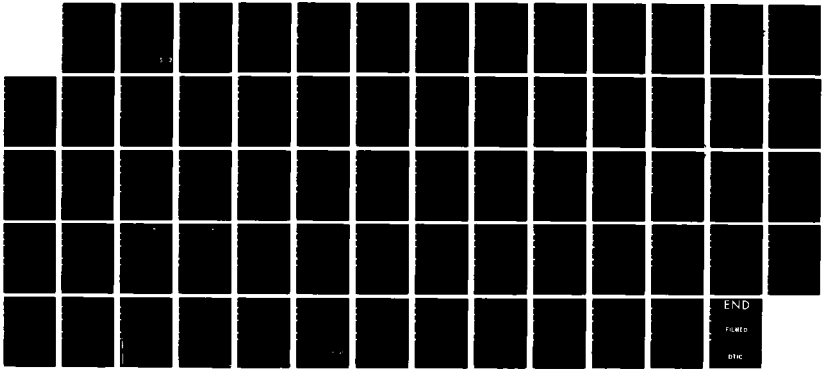

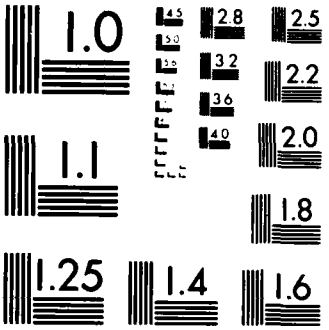

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Safety and Immunogenicity Testing of a Pilot Polysaccharide Vaccine Preparation to Pseudomonas Aeruginosa

Annual Report

Gerald B. Pier, Ph.D.

September 1, 1982

(For period 2 August 1981 to 15 August 1982)

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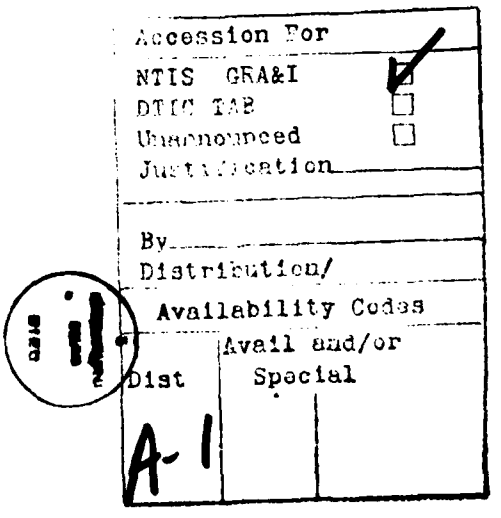
FOREWORD

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

For the protection of human subjects the investigator(s) have adhered to policies of applicable Federal Law 45CFR46.



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A. Contract Background

The principal investigator was awarded a contract from the USAMRDC to run from 1 March 1979 to 31 December 1982. The scope of this contract included preparation and testing of a pilot vaccine to Pseudomonas aeruginosa infections including human phase 1 trials, development of assays for measuring the antibody response of immunized and infected individuals, testing of the immunogenicity of the vaccine product in animals, assessment of the material in compromised animal host models as a protective agent, assessment of cell mediated protective mechanisms induced by the vaccine, including in vitro cellular studies, determinations of the genetic basis of response to the vaccine in inbred mouse strains, and assessment of the role of lipopolysaccharide (LPS) as an immuogenic or biologically active component of the vaccine. The vaccine is a high molecular weight polysaccharide (PS) material isolated from the outer cell surface or cultural supernates of P. aeruginosa (1). Simlar types of polysaccharides have been shown to be effective vaccines for a number of bacterial infections, such as meningitis caused by Neisseria meningitidis and pneumonia caused by Streptococcus pneumoniae. Since P. aeruginosa infections are common complications of wound and burn injuries that occur in military combat, this high molecular weight PS product is being developed as a potential preventative measure for these infections.

B. Progress of Vaccine Testing

1. Vaccine Production

This contract has centered around the production of a high molecular weight polysaccharide (PS) fraction from cultural supernatants of the organism Pseudomonas aeruginosa for use as a vaccine. Our method of preparation has been described in publications (1,2,3), and in previous annual reports submitted to the USAMRDC. The contract for the current year involves continued production of PS vaccines. Previous work was done with the immunotype 1 strain of P. aeruginosa. This year vaccine for testing in humans was to be produced from two additional immunotypes (immunotypes 2 and 3) of P. aeruginosa. These vaccines were to be produced in a manner appropriate for phase I and phase II human trials for safety and immunogenicity. The contract called for production of serologically active, immunogenic, non-toxic vaccine by the principal investigator at the Channing Laboratory; the final bottling of the vaccine in a form suitable for human use was to be performed by the Massachusetts State Biologic Laboratory. The contract also called for periodic testing of the serologic activity and immunogenicity in mice of previously prepared lots of vaccine in order to assess stability over time of these two biologic properties. In addition, the contract called for continued monitoring of the production procedure in order to ascertain that the PS vaccine was in as pure a form as possible.

During the course of the current year, we discovered that a component of the vaccine was not in fact a bacterial product, but was a product from the media. This component was, however, a polysaccharide molecule, identical in physical and biochemical properties to the polysaccharide of interest in the vaccine. The media component was identified as a mannan, a polymer of mannose residues similar to that produced by strains of

yeast. Although yeast extract is not a component of the media, this mannan appears to be part of one of the protein components of the media. Studies indicated that the mannan was not immunogenic in persons immunized with a PS vaccine from the IT-1 strain of P. aeruginosa. We have chosen to eliminate this media contaminant by chromatography of PS vaccines over an affinity gel, Concanavalin A covalently bound to Sepharose 4B (Con A-Sepharose). The Con A lectin binds the mannan component while allowing the PS vaccine to proceed through the column. The unbound fraction consists of the serologically active, immunogenic portions. Previous reports that suggested that the mannan component may be critical to the serologic activity and immunogenicity of the PS vaccine did not hold up under more careful scrutiny. Chromatography conditions of the PS vaccine over the Con A-Sepharose gel were found to affect the serologic activity and immunogenicity of the PS. When these conditions were modified, we could recover serologically active, immunogenic PS vaccine without the mannan component (Table 1). All lots of vaccine currently produced are either being passaged over the Con A-Sepharose affinity chromatography gel to remove the mannan component, or are being prepared from alternate media which lack this component (as well as other high molecular weight components). Confirmation that the vaccine prepared from Con A-Sepharose passed material was composed of products of a bacterial origin was obtained by preparing vaccine in media that had been passaged through ultrafiltration membranes (molecular weight cut off of 10,000) to eliminate high molecular weight components. Table 2 documents the monosaccharide composition of PS vaccine from IT-1 P. aeruginosa. This table shows that the monosaccharide composition of material obtained from Con A-Sepharose passed vaccine was very similar to material obtained from vaccine prepared from organisms grown in media passed through ultrafiltration membranes to remove high molecular weight components. However, passage of media through ultrafiltration membranes severely reduces the growth of the organism to a lower final concentration, with a subsequent reduction in yield of PS vaccine. In addition, low levels of mannan component was still present in these vaccines obtained from ultrafiltered media. Thus, a Con A-Sepharose chromatography step was still required. We are currently attempting to test other media lacking high molecular weight components in an effort to find a medium where the organisms can grow to a high level and produce a sufficient quantity of vaccine. If this cannot be accomplished, we still have the currently used option of growing organisms in media with mannan and passaging the vaccine over the Con A-Sepharose affinity chromatography gel to remove the mannan components. No other media components appear to be present in the final vaccine product prepared this way, as shown by the data in Table 2 and by other chemical analyses.

During the current contract year we have produced PS vaccine from IT-1, IT-2, and IT-3 P. aeruginosa, all lacking the mannan component, for testing in phase I and phase II human trials. Appendices A, B, and C contain the details of the production of these high molecular weight PS vaccines. These appendices also contain a summary of the biochemical and physical properties of the vaccine relating to their production. (Note: In these production protocols there are appendixes referred to which contain raw data sheets that are the daily log of the procedures used to prepare the vaccine. These are not included because they are both cumbersome and difficult to reproduce.) We have also submitted to the Food and Drug Administration (FDA) a notice of amendment to our

Investigational New Drug (IND) No. BB-IND-1495. These amendments reflect the changed production protocol, incorporating the Con A-Sepharose affinity chromatography step, for production of high molecular weight PS vaccine from P. aeruginosa. We have also included in this amendment the detailed protocol for the production of PS vaccine from the immunotype 3 strain of P. aeruginosa, which has not been previously submitted. These letters and amendment notices are all included in Appendices A, B, and C of this Annual Report.

Periodic testing of the serologic activity and immunogenicity of previously prepared vaccine has been undertaken only for products containing the mannan. The results of testing of the previously prepared lot of vaccine from IT-1 P. aeruginosa indicated that the material retained serologic activity and immunogenicity in animals over a one year period (see Appendix D, letter dated May 19, 1982). After discovery of the mannan contaminant, this lot of vaccine was recovered from the vaccine bottles, passaged over a Con A-Sepharose column to remove the mannan component, and tested for serologic activity, presence of mannan component, and immunogenicity in mice. The results indicate that after storeage for 14 months at -20°C, this material was serologically active and immunogenic in mice after removal of the mannan component. Therefore, it appears that storeage of the vaccine in individual aliquotes at -20°C is likely to result in a stable product for a minimum period of one year.

2. Phase I Human Trials

To date, phase I trials on the safety and immunogenicity of high molecular weight PS from P. aeruginosa have only been undertaken utilizing the IT-1 PS vaccine. Appropriate notification of the Bureau of Biologics of the FDA, the Human Use Review Office of the USAMRDC, and the Brigham and Women's Hospital Human Utilization Committee have been undertaken. In addition, the Channing Laboratory has established its own Vaccine Committee, chaired by Dr. E.H. Kass, Director of the Channing Laboratory, to oversee these studies. This Committee is consulted prior to the initiation of any vaccine studies on either new types of vaccines, or new phase I or phase II trials in humans. To date we have immunized 42 persons with the vaccine from IT-1 PS. Previous results on the immune responses in a radioactive antigen binding assay (RABA) and an opsonophagocytosis assay have been reported. During the current year we have continued to monitor the maintenance of elevated antibody levels over time, and determined which immunoglobulin classes were found in these immune sera following immunization. The purpose of this determination has been to assess which of the three major serum immunoglobulin isotypes (IgG, IgM, and IgA) were elicited in reponse to the vaccine. Further tests were done to determine which of these immunoglobulin isotypes was the most effective opsonin for mediating phagocytic killing of the organism, and which of the major serum phagocytic cell type (polymorphonuclear leukocyte, or mononuclear cells) could interact with the individual immunoglobulin isotypes for phagocytic killing. These results are presented in the following tables, and in the preprint of a manuscript submitted for publication documenting these results. Table 3 shows the duration of the immune response to the P. aeruginosa IT-1 PS vaccine. These data indicate that elevated antibody titers were still noted 16-21 months postimmunization. These levels were significantly ($p<.01$) elevated over preimmune levels, and suggest that a single dose of the

vaccine may be sufficient to maintain elevated titers of antibody to this antigen for long periods of time. Table 4 shows the distribution of immunoglobulin isotypes found in P. aeruginosa IT-1 PS vaccinates. Interestingly, we found that the relative proportions of IgG, IgM, and IgA did not change following immunization. However, the quantitative levels increased markedly (Table 3). IgG was found to be the predominant immunoglobulin isotype in almost all sera, whereas IgM and IgA were also found to be present in significant proportions. When these various immunoglobulin isotypes were separated into purified populations of IgG, IgM and IgA, then interacted with peripheral blood leukocytes and complement, it was found that each immunoglobulin could opsonize organisms for phagocytic killing in the presence of complement. Deletion of complement (C') from the reaction mixture reduced all of the IgM mediated phagocytic killing (Figure 1). Phagocytic killing mediated by IgA was unaffected by the presence of complement. This is consistent with the fact that this immunoglobulin isotype does not fix complement. Deletion of complement from purified IgG reduced the phagocytic killing from about 95% of the input inoculum to 75%. This was the approximate degree of reduction seen when whole sera was used, indicating that IgG was the major opsonin in the whole sera. When phagocytic cell populations were separated into purified populations of polymorphonuclear (PMN) leukocytes and mononuclear cells, and then reacted with the purified immunoglobulin isotypes, it was found that the different immunoglobulin isotypes, with and without complement, could interact with either PMN or mononuclear cells for phagocytic killing. Table 5 shows the opsonophagocytic killing of live IT-1 P. aeruginosa organisms mediated by purified serum immunoglobulin isotypes, with and without complement, in the presence of isolated PMN and mononuclear cells. With PMNs, IgG, IgA, and IgM plus complement all had about the same degree of killing of the input inoculum. Deletion of complement from the reaction mixture severely reduced the IgM mediated killing, further indicating that this immmunoglobulin isotype had an absolute requirement for complement in order to mediate phagocytic killing. The deletion of complement from the IgG reaction mixture reduced the killing by 18%. Interestingly, deletion of complement from the IgA reaction mixture reduced phagocytic killing by about 36%. This was not consistent with the inability of IgA to activate complement. The explanation for this appears to lie in the fact that in this experimental protocol, the organisms were opsonized with the IgA at 4°C for 30 min and washed prior to the addition of phagocytic cells and complement (or media in place of complement when complement was to be deleted). In cases such as these, it has been noted that the IgA aggregates on the surface of the organism can activate the alternative pathway of complement, and deposit the complement component, C3b, on the surface of the bacterial cell. The PMN phagocyte has receptors for C3b, and this likely explains the potentiation of IgA mediated killing in the presence of complement. When mononuclear cells were used as a source of phagocytic cells, again IgG, IgA and IgM all showed approximately equal killing levels in the presence of complement. Deletion of complement from this reaction mixture again had the most pronounced affect on IgM mediated killing, such that IgM in the absence of complement was poorly able to opsonize live P. aeruginosa for phagocytic killing. The IgG mediated killing was reduced by about 15% by the deletion of complement, whereas in this experiment IgA mediated killing was only slightly reduced (8%) in the absence of complement. In this system IgA was found to be the best opsonin for

mediating phagocytic killing by mononuclear cells. These data are consistent with other data that suggest that mononuclear cells and IgA can participate in phagocytic killing of extracellular organisms (4,5). In summary, the results from studies on the human immune response to the IT-1 PS P. aeruginosa vaccine indicate that the antibody response is long lived (maintained up to 21 months) and the principal immunoglobulin isotype elicited is IgG. All three major serum immunoglobulin isotypes could participate in opsonizing organisms for phagocytic killing by cells.

One final study also conducted was to assess whether the IgA elicited by vaccination participated in inhibition or blocking of phagocytic killing of these organisms. However, as noted above (Table 5) IgA was able to promote phagocytic killing, and not block it, indicating that vaccination of persons with the P. aeruginosa PS vaccine is not likely to result in a state of increased susceptibility due to circulating IgA. Such blocking antibodies have been described for other organisms (6,7).

Initiation of phase I safety and immunogenicity testing of the IT-2 and IT-3 PS vaccines has not commenced as of the date of this report. However, it is expected that these studies will commence before the end of the current contract year (December 31, 1982). We are currently awaiting modification of our contract from the USAMRDC to permit testing of these products in humans, as well as a meeting of the Vaccine Committee of the Channing Laboratory for final approval. Approval from the Brigham and Women's Hospital Human Use Committee is appended (Appendix E).

3. Initiation of Vaccine Studies in Selected At-Risk Patient Populations

The current contract calls for initiation of studies of the IT-1 PS vaccine in burn and trauma patients at the Brigham and Women's Hospital and the Massachusetts General Hospital. Because of the problems noted above on the presence of a media component in the vaccine, all previously prepared lots of vaccine are no longer being utilized for human studies. However, we are currently awaiting final approval of the Channing Laboratory Vaccine Committee to begin the studies of the IT-1 PS vaccine in burn and trauma patients. The purpose of these studies will be to determine if the injury received by these patients affects their ability to mount an immune response to this vaccine. These studies should commence prior to the end of the current contract year.

C. Analysis of the Immunologic Response to PS Vaccination in Mice

Mice have proven to be a good animal for assessing the immunogenicity of PS vaccines. A lot is known about murine immunology and this has provided us with an opportunity to use mice as experimental animals for studies that we hope to eventually perform in humans. During the current contract year we have done the following:

1) Documentation of cell mediated immune responses following PS vaccination.

2) Assessment of the in vitro mitogenic responses to PS antigens in murine lymphocytes.

1) We have investigated the ability of PS vaccine to induce cell mediated immune responses in mice. Cell mediated immunity is not generally thought to be an important component of immunity to extracellular bacterial pathogens such as P. aeruginosa. However, increasing evidence has indicated that this form of immunity may be important to protection against the certain aspects of disease caused by extracellular bacterial pathogens (8). During the course of the current contract year we have documented that immunization of inbred Balb/c mice with the IT-1 PS plus the cytotoxic drug, vinblastine, results in an immunity that appears to be cell mediated. These results are documented in the appendixed (Section H) manuscript. A summary of these data is as follows:

Immunization of the Balb/c mice with a normally nonimmunogenic, nonprotective dose of IT-1 PS (1 ug) plus 75 ug of the cytotoxic drug vinblastine, afforded protection to live organism challenge with the homologous strain (IT-1 P. aeruginosa). The kinetics and serotype specificity of immune response indicated that an active immunization had occurred. This was shown by the fact that immunity took 5-6 days to develop, and was specific for the IT-1 strain of P. aeruginosa. When serum antibody levels were analyzed from mice given the PS drug regimen utilizing the RABA, no immune response to the IT-1 PS could be detected. Furthermore, attempts to transfer the immunity from immune to nonimmune mice utilizing sera also yield a negative result. However, when immunity was transferred utilizing spleen cells from immune animals the recipients were protected against live organism challenge. The phenotype of a cell expressing this immunity was found to be a T cell, as evidenced by the ability of antisera directed to the T cell antigen, Thy-1.2, to remove the ability of spleen cell populations to transfer immunity. These studies also showed that congenially athymic (nude) mice also became immune to a dose of PS plus vinblastine. This was an unexpected finding since nude mice lack functional T cells. However, in these mice it was found that this immunization schedule of PS and drug resulted in the development of serum antibodies. Interestingly, the nude mice also developed antibody to a high dose of the antigen in the absence of vinblastine, indicating that this antigen can be immunogenic in the absence of T cells. Thus, we have established a model for looking at the role of cell mediated immunity to live organism challenge against P. aeruginosa.

We have also assessed the role of cell mediated immunity in protection against P. aeruginosa infection utilizing burned mice. In this study we found that vinblastine by itself was able to significantly protect the mice to live organism challenge. The best protective immunity was seen in animals given the PS and drug (Table 6), but significant protection was noted with vinblastine alone. This appears to be based on the fact that vinblastine can enhance nonspecific immunity. The protocol used in the burn mouse experiments detects this enhanced nonspecific immunity, whereas the protocol utilizing challenge of intact animals with an intraperitoneal injection cannot detect this nonspecific enhanced immunity. However, these results do indicate that cell mediated immune effector

mechanisms may be important in protecting the burned animal from P. aeruginosa infection. These studies are currently being repeated to further fine tune the system to assess the potential role of specific cell mediated immunity in protection against P. aeruginosa infections following burning.

The above experimental protocols utilized low doses (nonimmunogenic without vinblastine) of PS vaccine. These doses do not elicit antibody responses. Assessment of the ability of spleen cells to transfer immunity from mice that are given a dose of PS that does result in generation of serum antibody were not successful. The basic finding showed that we could transfer immunity with spleen cell populations, but a high number (10^8) of immune spleen cells were needed to transfer this immunity. Because of the difficulty in obtaining this large number of immune spleen cells, and the variability in their ability to transfer immunity, we have not conclusively demonstrated the development of cell mediated immunity in animals given a dose of PS vaccine that also results in antibody. These results do not question the role of antibody in protecting animals and humans against P. aeruginosa infections, but merely suggest that cell mediated immunity may have an auxillary or complimentary role in the overall immune picture to P. aeruginosa infections. The fact that the PS vaccine could induce a cell mediated immune type effector mechanism under certain experimental conditions further supports the idea that cell mediated immunity may be an important component to the overall immunity against P. aeruginosa infections.

2) Assessment of the in vitro mitogenic responses to PS in immune and nonimmune mice has recently been undertaken. Table 7 shows an enhanced mitogenic response of murine splenic lymphocytes to PS 7 days after an immunogenic dose (50 ug) of the vaccine was given. These results suggest that mitogenesis assays may allow us to document which cell types are important in generation of the immune response to the P. aeruginosa PS vaccine. The significance of these results would be the fact that cell mediated immunity, as noted above, may play an important role in the overall immunity to P. aeruginosa. Secondly, since IgG is known to be the most effective opsonin for P. aeruginosa cells (9,10), it is important to see if we can document the involvement of T cells in generation of PS immune responses. T cells are required for the production of IgG antibody, and the demonstration that T cells may be responding to PS vaccination would be an important correlate of the potential efficacy of PS vaccines being developed. Currently, we are assessing whether the mitogenic response observed against PS vaccine in the immune animals is being seen in the T cell and/or B cell population of lymphocytes. Once these studies have been completed, we will then determine whether or not the mitogenic response seen against PS vaccination is antigen specific (pauciclonal) or is a general stimulation of cells (polyclonal). A pauciclonal response would indicate that specific immune cells are responding, whereas a polyclonal response would indicate that numerous cells are responding, presumably due to lymphokines secreted by specifically reactive cells.

D. Progress of Testing of PS Vaccine Efficacy in the Animal Model

We have been working with Dr. Matt Pollock of the Uniformed Services University of the Health Sciences in assessing the efficacy of PS vaccine in a burn mouse model (11). This model involves immunizing the animals with the PS vaccine, followed 7 days later by an alcohol flame burn of the mouse. The animal is then inoculated with varying doses of the P. aeruginosa challenge strain. We have previously documented (Third Annual Report) the ability of the IT-1 PS vaccine to significantly elevate the number of P. aeruginosa organisms needed to kill a mouse following immunization with this vaccine. We have also shown that passive transfer to mice of rabbit antibody made to the IT-1 PS vaccine was effective in protecting the mice from challenge. During the current contract year we have extended these findings to the IT-2 PS vaccine (Table 8). These findings showed that active immunization of C3H/FeJ mice with the IT-2 PS vaccine resulted in a highly significant ($p<.01$) protection against live organism challenge. Passive transfer of antisera was also effective in protecting the burned mouse. In addition we showed (Table 8) that IT-1 PS and IT-2 PS could protect mice against challenge with heterologous strain of live organism. This showed that the IT-1 PS and IT-2 PS were cross-immunogenic in these mice. We have shown this to be true in outbred mice (Pier, G.B. manuscript submitted), and have now extended these findings to this strain of inbred mice and in the burn mouse model. Assessment of the protective efficacy of the IT-3 PS vaccine prepared for human use will be performed prior to the end of the current contract year.

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F. TABLES

Table 1

Immune Response of Mice to P. aeruginosa IT-1 PS Vaccine Before and After Removal of the Medium Mannan Component by Con A-Sepha se Affinity Chromatography

Immunogen	Activity		Ng antigen bound/100 ul sera[c]	
	Serologic[a]	Mannan[b]	Preimmune	Postimmune
IT-1 PS Lot VL-12 Pre Con-A Sepharose	+	+	9.0 ± 4.0	58.1 ± 15.0
IT-1 PS Lot VL-12 Post Con-A Sepharose	+	-	15.0 ± 3.3	80.6 ± 14.4

[a]Serologic activity determined by formation of precipitin line in immunodiffusion gel against rabbit antisera raised to IT-1 P. aeruginosa organisms. Antigen at 1 mg/ml.

[b]Presence of mannan determined by formation of precipitin live in immunodiffusion gel against Concanavalin A (5mg/ml).

[c]Mice immunized with 50 ug of PS in 0.5 ml saline. 7 days later mice were bled for postimmune sera and the increase in ng of antigen bound per 100 ul of sera determined in the RABA. Results are expressed as the average of four mice ± the standard error of the mean.

TABLE 2

Monosaccharide Analyses of 3 Lots of IT-1 PS Vaccine

	Lot Of IT-1 PS		
Monosaccharide	VL-12 With Mannan	VL-12 Con A-Sepharose Passed (No Mannan)	VL-14 Prepared From Ultrafiltered Medium
arabinose	7.3%	24%	22%
rhamnose	3.4	24.5	26
mannose	61.5	0	trace
galactose	20	30	32
glucose	8	9	7
xylose	trace	3	3
N-acetyl glucosamine	trace	11	10

TABLE 3

Duration Of Immune Response To *P. aeruginosa* IT-1 PS Vaccine

Antibody level (ug/ml) at			
Preimmune	Day 28	6-9 months	16-21 months
n=30	n=30	n=30	n=11*
9.7 ± 10.0†	76.5 ± 80.8	49.8 ± 49.3	72.2 ± 55.0

*11 of 30 persons available at 6 months post-immunization were also available at 16-21 months for antibody determinations.

†Represents average level of antibody in ug/ml ± standard deviation.

TABLE 4

Immunoglobulin Isotypes Found In *P. aeruginosa* IT-1 PS Vaccinates

Sera	No. Vaccinates*	Dosage of Vaccine (ug)	Percent of Total Antigen Bound		
			IgG	IgM	IgA
Preimmunization	12	250	54.7	16.6	28.6
Post-immunization, day 28	13	250	48.6	11.6	39.7
Post-immunization 6 months	10	250	48.7	12.0	39.3
Preimmunization	11	150	49.1	29.6	21.3
Post-immunization, day 28	8	150	56.5	28.0	15.5
Post-immunization 6 months	10	150	55.4	28.3	16.3
Preimmunization	2	75	59.8	12.2	18.1
Post-immunization, day 28†	2	75	37.1	11.4	51.4
Preimmunization	3	50	53.7	5.8	37.8
Post-immunization, day 28†	3	50	58.3	16.6	23.8

*No. of vaccinates varies due to sufficient antibody binding to detect Ig isotypes and availability of sera for study.

†6 month serum samples were not available from enough persons given 50 and 75 ug to study.

TABLE 5

Opsonophagocytic Killing Of IT-1 *P. aeruginosa* Mediated By Purified Serum Immunoglobulin Isotypes, With And Without Complement, In The Presence Of Isolated Peripheral Blood Polymorphonuclear (PMN) Leukocytes And Mononuclear Cells

Cell Type	Components added	Percentage Kill of Initial Inoculum at 60 Min
PMN	IgG, C'	77
	IgG	59
	IgA, C'	78
	IgA	42
	IgM, C'	71
	IgM	15
	C'	0*
	Media	6
Mononuclear	IgG, C'	55
	IgG	40
	IgA, C'	66
	IgA	58
	IgM, C'	49
	IgM	35
	C'	0*
	Media	0*

*0% kill indicates growth in tube during 60 min incubation period.

TABLE 6

Role of Cell Mediated Immunity in Protecting Burned Mice From Challenge With *P. aeruginosa*

Immunized With	Dose (ug)	LD_{50} at 10 days[a]
IT-1 PS plus Vinblastine	1 75	1.0×10^{7}[b]
IT-1 PS	1	2.6×10^{6}
Vinblastine	75	6.5×10^{6}
PBS	-	6.5×10^{4}

[a] LD_{50} calculated by a method of Reed and Muench. See Reference 11 for details of protocol.

[b] All three groups were statistically significantly different ($p<.05$) than the LD_{50} for the group receiving PBS only.

Mitogenic Response of Spleen Cells From C3H/ANF Mice

Mitogen	Dose (ug/ml)	SI[a] immune cells (50 ug PS)	non-immune cells (saline only)
IT-1 PS			
	500	12.2	3.4
	250	13.3	2.3
	100	7.8	1.9
	50	9.1	2.2
	10	2.1	1.1
	5	1.1	1.9
	1	1.1	0.9
Concanavilin A	4	29.2	23.4
E. coli 055 LPS	25	21.2	22.3
P. aeruginosa type 1 LPS	25	28.2	22.3

[a] SI, stimulation index: $\frac{\text{cpm incorporated into stimulated culture}}{\text{cpm incorporated into unstimulated culture}}$

TABLE 8

Protection of Burned Mice from Challenge with IT-1 and IT-2 P. aeruginosa Following Immunization with Three 50 ug Doses of IT-1 and IT-2 PS

Immunogen	Challenge Strain	LD_{50} at 10 days[a]
IT-1 PS	IT-1	4.4×10^{7}[b]
	IT-2	2.2×10^{6}
IT-2 PS	IT-1	1.1×10^{8}
	IT-2	3.5×10^{7}
BSA	IT-1	1.8×10^{3}
	IT-2	3.5×10^{4}

[a] LD_{50} calculated by a method of Reed and Muench. See Reference 11 for details of protocol.

[b] All immunized mice were protected to a significant ($p<.05$) degree over the protection seen with BSA immunization only.

G. APPENDICES

APPENDIX A

Production of Pseudomonas aeruginosa high molecular weight polysaccharide from an immunotype 1 strain (05139).

Production of P. aeruginosa high molecular weight polysaccharide from immunotype 1 strain (05139)

Introduction: High molecular weight (mw) polysaccharides (PS) have been found in the extracellular fractions of P. aeruginosa cultures (1,2). These polysaccharides appear to be immunogenic, non-toxic forms of the lipopolysaccharide (LPS) "O" side chain polysaccharide. High mw PS share serologic specificity with the "O" side chain polysaccharide isolated from intact LPS by mild acetic acid hydrolyses, but differ from "O" side chains by molecular size, monosaccharide composition, and immunogenicity in animals (1,2,3).

A previous preparation of high mw PS from an immunotype 1 (T-1) strain of P. aeruginosa has been shown to be safe and immunogenic when given to healthy humans at doses as high as 250 ug. The accompanying immune response was noted at 2 weeks post immunization and lasted up to 6 months post immunization (4). The antibody elicited was capable of opsonizing live P. aeruginosa T-1 for phagocytic killing by peripheral blood leukocytes.

During the course of studies on the production of high mw PS from other types of P. aeruginosa for testing in phase 1 and 2 human trials, we discovered that a component of the previously prepared vaccines came from the medium, trypticase soy broth (TSB). This component was identified as a mannan, a polymer of mannose residues, structurally similar to yeast mannans. Although yeast extract is not a component of TSB it was found to occur in the soy bean digest fraction of the TSB. This component was identified by its ability to react with Concanavalin A (Con A), a lectin with specificity for mannose residues. As a result of these findings, all previously prepared lots of P. aeruginosa vaccine that contained this component are no longer available for human use.

The following protocol describes the preparation of high molecular weight PS from an immunotype 1 strain of P. aeruginosa, 05139. This strain is the prototype immunotype 1 strain obtained from Dr. Myron Fisher of the Parke-Davis Company, Detroit, Michigan. Four lots were separately produced, designated VL-11, VL-12, VL-13, and VL-14. Each of these lots was processed in a very similar manner, prior to pooling together the serologically active, LPS free fractions for final removal of the mannan component by chromatography over a column of Con A-Sepharose.

The daily log of how each lot of high mw PS from T-1 was produced is appended to this protocol under Appendix A.

General protocol for production of high mw PS from T-1 P. aeruginosa:

A. Growth of starter culture and production culture.

1. A lyophilized stock culture was rehydrated, and a loopful of this suspension inoculated into 10 ml of TSB and onto a TSA plate for lots VL-13 and VL-14. For Lot VL-11 the lyophilized vial was rehydrated and used to directly inoculate the culture. For Lot VL-12 the original culture came from a TSA slant maintained in our laboratory with a live culture of the same T-1 P. aeruginosa strain 05139. The starter culture was incubated at 37° C for 21-72 hr. Morphology and purity were checked by Gram stain reaction and appearance of the growth on the plate. The serologic reaction of the strain was checked in an agglutination assay against standard typing antisera.

2. The 10 ml tube of TSB was inoculated into 30 L of TSB (3%) plus 1% sodium acetate. For Lot VL-14, this media was passed through PM 30 membranes (molecular weight cut off 30,000) in a TCE ultrafiltration apparatus, in an attempt to remove the mannan component. This was incubated 48-69.5 hr at 37° C with aeration (about 5L/hr) by sterile air from a compressed air tank.

3. At the end of the growth period, 10 ml of culture was removed, the purity checked by Gram stain reaction and a TSA plate inoculated. For Lots VL-13 and VL-14, three 1 ml aliquotes were made and frozen at -80° C. The next day the plate growth was checked for colonial appearance, Gram stain reaction and serotype reaction.

B. Processing of each lot:

4. For Lots VL-11, VL-13, and VL-14, the culture was killed by the addition of 50-150 gm of hexadecyltrimethyl ammonium bromide (Cetavalon). For Lot VL-12 the culture was killed by the addition of formalin to a final concentration of 1%, and the culture stored overnight at 4° C. The inactivated cultures were spun in the Sharples centrifuge and the supernate collected.

5. The culture supernate was filtered through 0.8 um and 0.45 um filters to remove residual bacteria. The supernates were then concentrated to about 1 L on a TCE ultrafiltration apparatus using PM-30 membranes. The desired portion was then recovered by addition of 4 volumes of alcohol (95% ethanol, EtOH). The alcohol precipitate was stored at 4° C for 24-96 hr.

6. The precipitate was recovered by centrifugation, redissolved in 210-600 ml sterile deionized water and a 10% solution of Cetavalon added to a 1% final concentration. The Cetavalon precipitated material was removed by centrifugation, and the supernate made isotonic by the addition of 10X phosphate buffered saline (PBS, final concentration: 0.1M phosphate, 0.15M NaCl). To this was added 4 volumes of 95% EtOH.

7. The EtOH precipitate was recovered by centrifugation and redissolved in 130-200 ml sterile deionized water. Step 6 was then repeated once.

8. The EtOH precipitate was redissolved in 1% acetic acid, the pH adjusted to 4.9-5.0 and the solution heated at 90-95° C for 18 hr. After cooling the precipitated lipids were removed by centrifugation.

9. The supernate material was tested in Ouchterlony immunodiffusion for serologic activity.

10. The material was then extracted 10 times with chloroform to remove lipids and other materials soluble in this solvent.

11. The recovered aqueous layer was then extracted with an equal volume of 90% phenol. The phenol and aqueous layers were separated by cooling (4° C, 18 hr) and centrifugation, and each phenol layer reextracted with water, and each aqueous layer reextracted with phenol.

12. Both the pooled aqueous and phenol layers were precipitated with alcohol, redissolved in PBS and tested for serologic activity in Ouchterlony immunodiffusion slides.

13. For all of the T-1 preparations the serologic activity desired was found in the aqueous phase, and this material was then applied to the 5.0 x 100 cm S-300 column.

14. The serologically active material was chromatographed on a Sephacel S-300 column reserved for exclusive production of vaccine. The washing and storeage of this column are documented in a log kept next to the column. Xerox copies of the pages relevant to the production of the type 1 vaccine are included in Appendix B. (Note - Lots VL-11 and VL-12 were processed on an identical but different vaccine column prior to keeping these records.) The high molecular weight fractions that eluted from the S-300 vaccine column were pooled and precipitated by the addition of four volumes of 95% EtOH.

15. After storeage at 4° C for 24-72 hours the EtOH precipitate was spun out, redissolved in approximately 100 ml of sterile distilled water and dialyzed against several changes of deionized water for 48 hours. The material was then filtered through 0.45 um filters and lyophilized to dryness. The resulting yield was weighed and the material tested for serologic activity in Ouchterlony immunodiffusion slides.

16. From each lot the following yields were obtained:

Lot VL-11, 3/6/81, 466.8 mg. Lot VL-12, 12/22/81, 175.1 mg. Lot VL-13, 3/6/82, 770.5 mg. Lot VL-14, 4/14/82, peak 1, 52.9 mg, peak 2, 47.8 mg. Each of these were the serologically active fractions.

17. For T-1 VL-13 three aliquotes were run over a Con A Sepharose column to remove the mannan component from this particular lot. The detailed report on the procedure used to remove the mannan component from this column is documented on the T-1 VL-13 protocol, and additional pages, following the T-1 VL-13 detailed protocol in Appendix A. In addition to the Con A Sepharose column two of the three aliquotes of T-1 VL-13 were also passed over a column of Sepharose 4B to which had been covalently linked castor bean lectin. This was because we found with an earlier lot of Type 1 PS that there was a component which reacted with this lectin, but was not involved in serologic activity, or in immunogenicity. However, this castor bean lectin-Sepharose 4B column was not used in any of the other runs after we had determined to our complete satisfaction that there was no material present in any of the lots of T-1 PS to be used for the vaccine that reacted with this lectin. Yields and serologic activities of the T-1 VL-13 passed over the Con A Sepharose and castor bean lectin columns are documented in Appendix A.

18. The final preparation from the last lot, T-1 VL-14, was obtained on 4/14/82. The recovered fractions were all stored in the dryed state at -20° C in vials placed in bags containing silica gel.

19. On 4/15/82 we reviewed the data for the four lots of type 1 high mw PS vaccine. The remaining materials from these fractions were tested for serologic activity in Ouchterlony immunodiffusion against antisera prepared in rabbits to T-1 whole organisms, and against the lectin Concanavalin A (Con A, 5 mg/ml) to determine the presence of mannan in each of the four lots. Reactivity against Con A was found in all four of the lots, including Lot VL-14, wherein the media had been passed through ultrafiltration membranes prior to growth in an attempt to remove the mannan component. The reactivity in the VL-14 fractions was not as strong as that observed in the others.

20. On 4/26/82 the following amounts of the various lots were pooled for removal of the mannan commmponent: (Lot VL-11, 77 mg; Lot VL-12, 25 mg; Lot VL-13, 22 mg; Lot VL-14, 47.8 mg (peak 2), total 171.8 mg. These materials were dissolved in 15 ml of Con A buffer (0.1 M acetate, 0.15 M NaCl, 0.001 M $MgCl_2$, $CaCl_2$, and $MnCl_2$, and 0.02% merthiolate).

21. This solution was placed on a column of 100 ml of Concanavalin A covalently bound to Sepharose. The maintenance and use of this column is also documented in a log kept close to the column. The page relevant to the production of this vaccine from this column is xeroxed and included under Appendix D.

22. One peak was recovered from the Con A column eluate (RI recording in Appendix D). This peak was collected, fractions 10-26 pooled, 400 ml of 95% EtOH added, the material placed at 4° C for 1 hour, the precipitate collected in the Sorvall centrifuge (8,000 rpm 10 min), redissolved in sterile phosphate buffered saline, and frozen at -20° C.

23. 4/28/82. The eluate from the Con A column consisting of vaccine material obtained from lots VL-11, VL-12, VL-13, and VL-14 were added to 176.2 mg of T-1 VL-13 previously passed over the Con A column.

24. The pooled high mw PS vaccine from T-1, purified of the mannan component, was placed on the S-300 vaccine column. The preparation and use of this column are documented in Appendix E. Three peaks were noted to elute from this column. The fractions in these peaks were pooled, precipitated by the addition of four volumns of 95% EtOH, the precipitates collected by centrifugation, redissolved in sterile deionized water, dialyzed against numerous changes of deionized water and lyophilized.

25. The resulting yield from the first peak (highest molecular weight) was 107.7 mg. A portion of this material was removed for testing in Ouchterlony immunodiffusion. The results indicated that this material reacted specifically with antisera prepared in rabbits to whole IT-1 P. aeruginosa cells. A 5 mg/ml solution of this material did not react with Concanavalin A (5 mg/ml) in immunodiffusion gels.

26. 75 mg of the T-1 high mw PS preparation were taken to Miss Leslie Wetterlow of the Massachusetts Public Health Biologic Laboratories for processing and bottling in 200 ug individual aliquotes.

27. In Appendix F are the records regarding the results of chemical tests for thimersol, bulk sterility tests, safety tests in guinea pigs, pyrogen tests in rabbits, final sterility tests, and identity and potency tests. Having passed all these tests satisfactorily, the vaccine was released to us on June 23, 1982. One 200 ug vial was removed and rehydrated with 200 ul of sterile saline for injection. This material was tested in Ouchterlony immunodiffusion against rabbit antisera raised to T-1 P. aeruginosa whole organisms and against the lectin, Concanavalin A (5 mg/ml). Results obtained on 6/25/82, diagramed in the laboratory record book of Diane Thomas, and photographed (Appendix F) showed that the vaccine reacted with the antisera to the T-1 P. aeruginosa organisms and gave a line of identity with standard T-1 polysaccharide at a 1 mg/ml. The vaccine did not react with Concanavalin A. A positive control of mannan reacted well with the Concanavalin A, giving a strong precipitin line.

28. The test for immunogenicity of the vaccine was performed in five C3H/HeJ mice. The mice were anesthetized with ether and bled from the retro-orbital plexus. They were then given 25 ug of Type 1, Lot 3, vaccine in 0.5 ml sterile saline via an intraperitoneal injection on 7/2/82. Seven days later on 7/9/82 the mice were again anesthetized and bled from the retro-orbital plexus. The serum was allowed to clot at 37° C for 1/2 hour and overnight at 4° C. A Farr test was set up as follows. 100 ul of the pre- and post-immunization sera were mixed with 50 ul of the Farr antigen, which is an intrinsically ^{14}C-labelled polysaccharide preparation, with a specific activity of 1.02 cpm/ng. The antigen and antibody were incubated at 37° C for 2 hours, then placed at 4° C in tightly stoppered tubes over the rest of the weekend. On 7/12/82 the Farr test was completed by adding 150 ul of saturated ammonium sulfate

to each of the test tubes, placing the tubes at 4° C for 1/2 hour, followed by addition of 0.5 ml of 50% ammonium sulfate. The precipitate was spun out, washed once in 0.5 ml of 50% ammonium sulfate, the wash poured off, the pellet allowed to dry, then dissolved in Protosol and liquiflor/toluene for counting in a scintillation counter. The results were obtained on 7/13/82 and are as presented in Appendix F.

29. An additional test wherein the molecular size of the material was determined on high pressure liquid chromatography columns indicated that this vaccine lot had a molecular weight of greater than 100,000.

30. Chemical analyses of the product are presented in Appendix G. The protein and nucleic acid contents were determined in a portion of the material not sent to the State Health Laboratory, because the presence of merthiolate in the final product interfers with these assays. These data indicate that the resultant antigen was composed principally of carbohydrate (about 83%) with low levels of contaminating nucleic acid and protein.

31. Analysis of the coagulation activity of the vaccine in the limulus amebocyte lysate assay indicated that it took 1,000 times more vaccine than LPS from T-1 P. aeruginosa to gel the limulus amebocyte assay, (and 500,000 times as much vaccine as E. coli LPS, Appendix G).

32. Description of final product - This is a freeze dried preparation of purified high molecular weight polysaccharide vaccine to P. aeruginosa T-1, prepared from 4 lots of material derived from Strain 05139, the prototype immunotype 1 strain according to the scheme of Fisher Devlin and Gnabski. Each vial contains 200 ug of the purified polysaccharide and sufficient merthiolate (Thimersol) to have a concentration of 1:30,000 when the product is rehydrated with 2 ml (100 ug/ml) of sterile saline.

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APPENDIX B

Production of Pseudomonas aeruginosa high molecular weight polysaccharide from an immunotype 2 strain (PBBH-9882-80).

Production of P. aeruginosa high molecular weight polysaccharide from immunotype 2 strain PBBH-9882-80

Introduction: High molecular weight (mw) polysaccharides (PS) have been found in the extracellular fractions of P. aeruginosa cultures (1,2). These polysaccharides appear to be immunogenic, non-toxic forms of the lipopolysaccharide (LPS) "O" side chain polysaccharide. High mw PS share serologic specificity with the "O" side chain polysaccharide isolated from intact LPS by mild acetic acid hydrolyses, but differ from "O" side chains by molecular size, monosaccharide composition, and immunogenicity in animals (1,2,3).

A previous preparation of high mw PS from an immunotype 1 (IT-1) strain of P. aeruginosa has been shown to be safe and immunogenic when given to healthy humans at doses as high as 250 ug. The accompanying immune response was noted at 2 weeks post immunization and lasted up to 6 months post immunization (4). The antibody elicited was capable of opsonizing live P. aeruginosa IT-1 for phagocytic killing by peripheral blood leukocytes.

During the course of studies on the production of high mw PS from other types of P. aeruginosa for testing in phase 1 and 2 human trials, we discovered that a component of the previously prepared vaccines came from the medium, trypticase soy broth (TSB). This component was identified as a mannan, a polymer of mannose residues, structurally similar to yeast mannans. Although yeast extract is not a component of TSB it was found to occur in the soy bean digest fraction of the TSB. This component was identified by its ability to react with Concanavalin A (Con A), a lectin with specificity for mannose residues. As a result of these findings, all previously prepared lots of P. aeruginosa vaccine that contained this component are no longer available for human use.

The following protocol describes the preparation of high molecular weight PS from an immunotype 2 strain of P. aeruginosa, PBBH-9882-80. This strain was isolated from the blood of a patient at the Peter Bent Brigham Hospital (now the Brigham and Women's Hospital) in 1980, and was shown to contain and produce the immunotype 2 specific PS. Four lots were separately produced, designated VL-6, VL-7, VL-8 and VL-9. Each of these lots was processed in a very similar manner, prior to pooling together the serologically active, LPS free fractions for final removal on the mannan component by chromatography over a column of Con A-Sepharose.

The daily log of how each lot of high mw PS from IT-2 was produced is appended to this protocol under Appendix A.

General protocol for production of high mw PS from IT-2 P. aeruginosa:

A. Growth of starter culture and production culture.

1. A frozen (-80° C) stock culture was thawed and a loopful of suspension inoculated into 10 ml of TSB and onto a TSA plate. This was incubated at 37° C for 22-24 hr. Morphology and purity were checked by Gram stain reaction and appearance of the growth on the plate. The serologic reaction of the strain was checked in an agglutination assay against standard typing antisera.

2. The 10 ml tube of TSB was inoculated into 30 L of TSB (3%) plus 1% sodium acetate. This was incubated 66-93 hrs at 37° C with aeration (about 5L/hr) by sterile air from a compressed air tank.

3. At the end of the growth period, 10 ml of culture was removed, the purity checked by Gram stain reaction and a TSA plate inoculated. Three 1 ml aliquotes were made and frozen at -80° C. The next day the plate growth was checked for colonial appearance, Gram stain reaction and serotype reaction.

B. Processing of each lot:

4. For lots VL-6 and VL-7 the culture was killed by addition of 310 ml of formalin, and the culture stored overnight at 4° C. For lots VL-8 and VL-9 the culture was killed by addition of 150 gm of hexadecyltrimethyl ammonium bromide (Cetavalon). The inactivated cultures were spun in the Sharples centrifuge and the supernate collected.

5. The culture supernate was filtered through 0.8 um and 0.45 um filters to remove residual bacteria. The supernates were then concentrated to about 1 L on a TCE ultrafiltration apparatus using PM-30 membranes. The desired portion was then recovered by addition of 4 volumes of alcohol (95% ethanol, EtOH). The alcohol precipitate was stored at 4° C for 24-96 hr.

6. The precipitate was recovered by centrifugation, redissolved in 240-360 ml sterile deionized water and a 10% solution of Cetavalon added to a 1% final concentration. The Cetavalon precipitated material was removed by centrifugation, and the supernate made isotonic by the addition of 10X phosphate buffered saline (PBS, final concentration: 0.1M phosphate, 0.15M NaCl). To this was added 4 volumes of 95% EtOH.

7. The EtOH precipitate was recovered by centrifugation and redissolved in 125-200 ml sterile deionized water. Step 6 was then repeated once.

8. The EtOH precipitate was redissolved in 1% acetic acid, the pH adjusted to 5.0-5.8 and the solution heated at 90-95° C for 18 hr. After cooling the precipitated lipids were removed by centrifugation.

9. The supernate material was tested in Ouchterlony immunodiffusion for serologic activity.

10. The material was then extracted 10 times with chloroform to remove lipids and other materials soluble in this solvent.

11. The recovered aqueous layer was then extracted with an equal volume of 90% phenol. The phenol and aqueous layers were separated by cooling (4° C, 18 hr) and centrifugation, and each phenol layer reextracted with water, and each aqueous layer reextracted with phenol.

12. Both the pooled aqueous and phenol layers were precipitated with alcohol, redissolved in PBS and tested for serologic activity in Ouchterlony immunodiffusion slides.

13. For all of the IT-2 preparations the serologic activity desired was found in the phenol phase, a unique property of this particular antigen.

14. The serologically active material was chromatographed on a Sephacel S-300 column reserved for exclusive production of vaccine. The washing and storeage of this column are documented in a log kept next to the column. Xerox copies of the pages relevant to the production of the type 2 vaccine are included in Appendix B. (Note - Lot VL-6 was processed on a different vaccine column prior to keeping these records.) The high molecular weight fractions that eluted from the S-300 vaccine column were pooled and precipitated by the addition of four volumes of 95% EtOH.

15. After storeage at 4° C for 24-72 hours the EtOH precipitate was spun out, redissolved in approximately 100 ml of sterile distilled water and dialyzed against several changes of deionized water for 48 hours. The material was then filtered through 0.45 um filters and lyophilized to dryness. The resulting yield was weighed and the material tested for serologic activity in Ouchterlony immunodiffusion slides.

16. From each lot the following yields were obtained:

Lot VL-6, 82.9 mg. Lot VL-7, 39.1 mg. Lot VL-8, 112.8 mg. Lot VL-9, 43.6 mg.

Each of these were the serologically active fractions.

17. The final preparation from the last lot, T-2 VL-9, was obtained on 4/8/82. The fractions were stored in the dryed state at -20° C in vials placed in bags containing silica gel.

18. On 4/13/82 we reviewed the data for the four lots of type 2 high mw PS vaccine. These had been tested in Ouchterlony immunodiffusion against Con A to determine the presence of mannan in each of the four fractions. Reactivity against Con A was found in the VL-6 and VL-8 lots (data in Mark Cohen's book 4/9/82).

19. The following amounts of materials were pooled from the four lots: VL-6, 80 mg; VL-7, 38 mg; VL-8, 102 mg; VL-9, 45 mg; total, 265 mg. These were dissolved in 20 ml of Con A buffer (0.1 M acetate, 0.15 M NaCl, 0.001 M $MgCl_2$, $CaCl_2$, and $MnCl_2$, and 0.02% merthiolate).

20. This solution was placed on a column of 100 ml of Concanavalin A covalently bound to Sepharose. The maintenance and use of this column is also documented in a log kept close to the column. The page relevant to the production of this vaccine from this column is xeroxed and included under Appendix C.

21. Two peaks were noted to pass through the Con A column (RI recording in Appendix C). These peaks were collected, fractions pooled and directly placed into dialysis versus running deionized water for 36 hours.

22. The dialyzed material was then placed on the lyophilizer for overnight drying.

23. The resulting yield of 77.4 mg from the first peak was stored at -20° C. The second peak yield was 6.9 mg (4/21/82). A portion of the first peak (0.2 mg) was removed for testing in Ouchterlony immunodiffusion. The results indicated that this material reacted specifically with antisera prepared in rabbits to whole IT-2 P. aeruginosa cells. A 5 mg/ml solution of this material did not react with Concanavalin A in immunodiffusion slides.

24. 74.5 mg of the IT-2 high molecular weight PS preparation were taken to Miss Leslie Wetterlow of the Massachusetts Public Health Biologic Laboratories for processing and bottling in 200 ug individual aliquotes.

25. In Appendix D are the records regarding the results of chemical tests for thimersol, bulk sterility tests, safety tests in guinea pigs, pyrogen tests in rabbits, final sterility tests, and identity and potency tests. Having passed all these tests satisfactorily, the vaccine was released to us on June 3, 1982. One 200 ug vial was removed and rehydrated with 200 ul of sterile saline for injection. This material was then tested in Ouchterlony immunodiffusion against rabbit antisera raised to IT-2 P. aeruginosa whole organisms and against the lectin, Concanavalin A (5 mg/ml). Results obtained on 6/4/82, diagramed in the laboratory record book of Diane Thomas, and photographed (Appendix D) showed that the vaccine reacted with the antisera to the IT-2 P. aeruginosa organisms and gave a line of identity with standard IT-2 polysaccharide at a 1 mg/ml and 0.5 mg/ml concentration. The vaccine did not react with Concanavalin A. A positive control of mannan reacted well with the Concanavalin A, giving a strong precipitin line.

26. The test for immunogenicity of the vaccine was performed in four C3H/HeJ mice, 12 weeks old. The mice were anesthetized with ether and bled from the retro-orbital plexus. They were then given 25 ug of

type 2, lot 2, vaccine in 0.5 ml sterile saline via an intraperitoneal injection. Seven days later on 6/11/82 the mice were again anesthetized and bled from the retro-orbital plexus. The serum was allowed to clot at 37° C for 1/2 hour and overnight at 4° C. On 6/12/82 a Farr test was set up as follows. 100 ul of the pre and post immunization sera were mixed with 50 ul of the Farr antigen, which is an intrinsically ^{14}C-labelled polysaccharide preparation, with a specific activity of 0.98 cpm/ng. The antigen and antibody were incubated at 37° C for 2 hours, then placed at 4° C in tightly stoppered tubes over the rest of the week-end. On 6/14/82 the Farr test was completed by adding 150 ul of saturated ammonium sulfate to each of the test tubes, placing the tubes at 4° C for 1/2 hour, followed by addition of 0.5 ml of 50% ammonium sulfate. The precipitate was spun out, washed once in 0.5 ml of 50% ammonium sulfate, the wash poured off, the pellet allowed to dry, then dissolved in Protosol and liquiflor/toluene for counting in a scintillation counter. The results were obtained on 6/15/82 and are as presented in Appendix E.

27. An additional test wherein the molecular size of the material was determined on high pressure liquid chromatography columns indicated that this vaccine lot had a molecular weight of greater than 70,000.

28. Chemical analyses of the product are presented in Appendix F. The protein and nucleic acid content was determined prior to submission of the vaccine to the State Health Laboratory, because the presence of merthiolate in the final product interfers with the these assays. These data indicate that the resultant antigen was composed principally of carbohydrate (about 82%) with low levels of contaminating nucleic acids and protein.

29. Analysis of the coagulation activity of the vaccine in the limulus amebocyte lysate assay indicated that it took 100 times more vaccine than LPS from IT-2 P. aeruginosa to gel the limulus amebocyte assay (and 50,000 times as much vaccine as E. coli LPS).

30. Description of final product - This is a freeze dried preparation of purified high molecular weight polysaccharide vaccine to P. aeruginosa IT-2, prepared from 4 lots of material derived from Strain PBBH 9882-80, an immunotype 2 strain according to the scheme of Fisher, Devlin and Gnabski. Each vial contains 200 ug of the purified polysaccharide and sufficient merthiolate (Thimersol) to have a concentration of 1:30,000 when the product is rehydrated with 2 ml (100 ug/ml) of sterile saline.

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APPENDIX C

Production of Pseudomonas aeruginosa high molecular weight polysaccharide from an immunotype 3 strain (PBBH-12136-80).

Production of P. aeruginosa high molecular weight polysaccharide from immunotype 3 strain (PBBH 12136-80)

Introduction: High molecular weight (mw) polysaccharides (PS) have been found in the extracellular fractions of P. aeruginosa cultures (1,2). These polysaccharides appear to be immunogenic, non-toxic forms of the lipopolysaccharide (LPS) "O" side chain polysaccharide. High mw PS share serologic specificity with the "O" side chain polysaccharide isolated from intact LPS by mild acetic acid hydrolyses, but differ from "O" side chains by molecular size, monosaccharide composition, and immunogenicity in animals (1,2,3).

A previous preparation of high mw PS from an immunotype 1 (T-1) strain of P. aeruginosa has been shown to be safe and immunogenic when given to healthy humans at doses as high as 250 ug. The accompanying immune response was noted at 2 weeks post immunization and lasted up to 6 months post immunization (4). The antibody elicited was capable of opsonizing live P. aeruginosa T-1 for phagocytic killing by peripheral blood leukocytes.

During the course of studies on the production of high mw PS from other types of P. aeruginosa for testing in phase 1 and 2 human trials, we discovered that a component of the previously prepared vaccines came from the medium, trypticase soy broth (TSB). This component was identified as a mannan, a polymer of mannose residues, structurally similar to yeast mannans. Although yeast extract is not a component of TSB it was found to occur in the soy bean digest fraction of the TSB. This component was identified by its ability to react with Concanavalin A (Con A), a lectin with specificity for mannose residues. As a result of these findings, all previously prepared lots of P. aeruginosa vaccine that contained this component are no longer available for human use.

The following protocol describes the preparation of high molecular weight PS from an immunotype 1 strain of P. aeruginosa, 05139. This strain is the prototype immunotype 1 strain obtained from Dr. Myron Fisher of the Parke-Davis Company, Detroit, Michigan. Four lots were separately produced, designated VL-11, VL-12, VL-13, and VL-14. Each of these lots was processed in a very similar manner, prior to pooling together the serologically active, LPS free fractions for final removal of the mannan component by chromatography over a column of Con A-Sepharose.

The daily log of how each lot of high mw PS from IT-3 was produced is appended to this protocol under Appendix A.

General protocol for production of high mw PS from IT-3 P. aeruginosa:

A. Growth of starter culture and production culture.

1. A frozen (-80°C) culture of strain PBBH 12136-80 was thawed and a loopful of this suspension inoculated into 10 ml of TSB and onto a TSA plate for lots VL-1, VL-2, VL-3 and VL-4. The starter culture was incubated for 21 to 24 hr. Morphology and purity were checked by Gram stain reaction and appearance of the growth on the plate. The serologic reaction of the strain was checked in an agglutination assay against standard typing antisera.

2. The 10 ml tube of TSB was inoculated into 30 L of TSB (3%) plus 1% sodium acetate. For Lot VL-4, this media was passed through PM 30 membranes (molecular weight cut off 30,000) in a TCE ultrafiltration apparatus, in an attempt to remove the mannan component. This was incubated for 50-73 hr at 37° C with aeration (about 5L/hr) by sterile air from a compressed air tank.

3. At the end of the growth period, 10 ml of culture was removed, the purity checked by Gram stain reaction and a TSA plate inoculated. For Lot VL-4, three 1 ml aliquotes were made and frozen at -80° C. The next day the plate growth was checked for colonial appearance, Gram stain reaction and serotype reaction.

B. Processing of each lot:

4. For Lots VL-1, VL-2, and VL-4, the culture was killed by the addition of 150 gm of hexadecyltrimethyl ammonium bromide (Cetavalon). For Lot VL-3 the culture was killed by the addition of formalin to a final concentration of 1%, and the culture stored overnight at 4° C. The inactivated cultures were spun in the Sharples centrifuge and the supernate collected.

5. The culture supernate was filtered through 0.8 um and 0.45 um filters to remove residual bacteria. The supernates were then concentrated to about 1 L on a TCE ultrafiltration apparatus using PM-30 membranes. The desired portion was then recovered by addition of 4 volumes of alcohol (95% ethanol, EtOH). The alcohol precipitate was stored at 4° C for 16-24 hr.

6. The precipitate was recovered by centrifugation, redissolved in 240-600 ml sterile deionized water and a 10% solution of Cetavalon added to a 1% final concentration. The Cetavalon precipitated material was removed by centrifugation, and the supernate made isotonic by the addition of 10X phosphate buffered saline (PBS, final concentration: 0.1M phosphate, 0.15M NaCl). To this was added 4 volumes of 95% EtOH.

7. The EtOH precipitate was recovered by centrifugation and redissolved in 100-600 ml sterile deionized water. Step 6 was then repeated once.

8. The EtOH precipitate was redissolved in 1% acetic acid, the pH adjusted to 5.0-5.8 and the solution heated at 90-95° C for 18 hr. After cooling the precipitated lipids were removed by centrifugation.

9. The supernate material was tested in Ouchterlony immunodiffusion for serologic activity.

10. Lot VL-4 also showed reactivity against Concanavalin A (5 mg/ml) in immunodiffusion and was passaged over a Con A-Sepharose column (50 ml) to remove the mannan component present in this lot. The material that did not bind to the Con A column was pooled, precipitated by the addition of four volumns of alcohol, the precipitate collected and redissolved in PBS (75 ml). Insoluble material were removed by centrifugation (10,000 rpm for 15 min) and both the supernatant and pellet tested for reactivity against rabbit antisera prepared to IT-3 P. aeruginosa organisms and Con A (5 mg/ml). Both the supernatant and pellet fraction showed reactivity against both of these materials, indicating that the Con A chromatography step had not removed all of the mannan. (Note - Subsequent studies have shown that the insoluble material could be readily redissolved by the addition of EDTA. This is due to the complexing of calcium in the Con A chromatography buffer to a fraction of the polysaccharide.) Both the supernatant and pellet fractions from the Con A Sepharose column were processed in the subsequent steps, but as separate aliquotes.

11. The material (all lots) was then extracted with chloroform to remove lipids and other materials soluble in this solvent.

12. The recovered aqueous layer was then extracted with an equal volume of 90% phenol. The phenol and aqueous layers were separated by cooling (4° C, 18 hr) and centrifugation, and each phenol layer reextracted with water, and each aqueous layer reextracted with phenol.

13. Both the pooled aqueous and phenol layers were precipitated with alcohol, redissolved in PBS and tested for serologic activity in Ouchterlony immunodiffusion slides.

14. For all of the IT-3 preparations the serologic activity desired was found in the aqueous phase, and this material was then applied to the 5.0 x 100 cm S-300 column.

15. The serologically active material was chromatographed on a Sephacel S-3CO column reserved for exclusive production of vaccine. The washing and storeage of this column are documented in a log kept next to the column. Xerox copies of the pages relevant to the production of the type 3 vaccine are included in Appendix B. (Note - Lots VL-1 and VL-2 were processed on an identical but different vaccine column prior to keeping these records.) The high molecular weight fractions that eluted from the S-300 vaccine column were pooled and precipitated by the addition of four volumes of 95% EtOH.

16. After storeage at 4° C for 24-72 hours the EtOH precipitate was spun out, redissolved in approximately 100 ml of sterile distilled water and dialyzed against several changes of deionized water for 48 hours. The material was then filtered through 0.45 um filters and lyophilized to dryness. The resulting yield was weighed and the material tested for serologic activity in Ouchterlony immunodiffusion slides.

17. From each lot the following yields were obtained:

Lot VL-1, 3/6/81, 212 mg. Lot VL-2, 12/22/81, 340.5 mg. Lot VL-3, 3/6/82, 230.1 mg. Lot VL-4, 4/14/82, peak 1, 37.7 mg; peak 2, 69.2 mg. Each of these were the serologically active fractions.

18. Lots VL-1, VL-2, and VL-3 were processed on Con A Sepharose columns to remove the mannan component from these particular lots. Aliquotes of each were run over Con A Sepharose columns, since we had determined that we could only put a limited amount of mannan on each of these columns to avoid saturation. The columns were eluted with 0.1 M borate buffer (pH 5.0) containing 1% alpha-methyl mannose to remove the mannan component, then washed extensively with 0.1 M Tris, pH 8.0 with 0.5 M NaCl, followed by 0.1 M acetate, pH 5.0, containing 0.5 M NaCl, prior to reequilibration in the Con A buffer.

19. The final preparation from the last lot, T-3 VL-4, was obtained on 5/13/82. The recovered fractions were all stored in the dryed state at -20° C in vials placed in bags containing silica gel.

20. On 6/1/82 we combined fractions from all four of the IT-3 P. aeruginosa high mw PS to prepare the IT-3 PS vaccine. This was designated IT-3 PS pool. The materials used to make up IT-3 PS pool are documented in Appendix C, which contains the daily log of the steps performed to prepare the final IT-3 vaccine. On 6/1/82 this material was placed on the 5.0 x 100 cm Sephacel S-300 vaccine column. The use, washing and storeage of this column is documented also in Appendix C.

21. Two peaks were observed to elute in the high molecular weight range from this column. The fractions composing these two peaks were pooled and precipitated by the addition of four volumns of alcohol. The precipitate was collected by centrifugation, dried and redissolved in sterile deionized water, placed into dialysis against 4L deionized water for each peak, the water was changed once and then the material lyophilized. The following yields were obtained: peak 1 - 174.4 mg; peak 2 - 157.5 mg.

22. These two peaks were tested in immunodiffusion against the lectin Con A (5 mg/ml) and rabbit antisera raised to IT-3 P. aeruginosa organisms. Results indicated that both peaks reacted with the antisera as well as the Con A indicating that the Con A Sepharose column had not removed all of the mannan component.

23. A new Con A Sepharose column was poured (6/6/82) and 20 mg of the IT-3 high mw PS vaccine, peak 2, placed on this column to determine if the material reacting with the Con A lectin was part of the IT-3 PS molecule or if it could be separated from the serologically active component. The material eluting from this column was pooled together, precipitated by the addition of 4 volumes of alcohol, the precipitate collected by centrifugation, redissolved in saline and EDTA, and tested in immunodiffusion gels for reactivity with the rabbit antisera to whole organisms in Con A.

24. 6/7/82. The eluted material was found to give a precipitin line in immunodiffusion gels with rabbit antisera to whole organisms but did not give any line against the Con A (5 mg/ml) lectin, indicating that the IT-3 PS itself does not react with Con A.

25. 6/7/82. 174 mg of the IT-3 PS vaccine (peak 1) was placed on the newly poured Con A Sepharose column (100 ml). The use and preparation of this column is documented in Appendix C. The material eluting from this column (fractions 23-47) was pooled and placed directly into dialysis versus running deionized water for 24 hr.

26. 6/8/82. The dialyzed material was removed, filtered, sterilized through 0.45 um membrane and lyophilized.

27. 6/9/82. The resulting yield was 148.9 mg. Testing of a 2 mg/ml solution in immunodiffusion versus antisera raised to whole organisms and to the lectin Con A showed that this material reacted with the antisera prepared to whole organisms, and gave a line of identity with previously prepared IT-3 PS. The material did not react with the Con A lectin, indicating that the mannan component had been successfully removed.

28. 80 mg of IT-3 high mw PS preparation were taken to Ms. Leslie Wetterlow of the Massachusetts Public Health Biologic Laboratories for processing and bottling in 250 ug individual aliquotes.

29. In Appendix D are the records regarding the results of chemical tests for thimersol, bulk sterility tests, safety tests in guinea pigs, pyrogen tests in rabbits, final sterility tests, and identity and potency tests. Having passed all these tests satisfactorily, the vaccine was released to us on August 2, 1982. One 250 ug vial was removed and rehydrated with 250 ul of sterile saline for injection. This material was tested in Ouchterlony immunodiffusion against rabbit antisera raised to IT-3 P. aeruginosa whole organisms and against the lectin, Concanavalin A (5 mg/ml). Results obtained on 8/3/82, diagramed in the laboratory record book of Diane Thomas, and photographed (Appendix F) showed that the vaccine reacted with the antisera to the IT-3 P. aeruginosa organisms and gave a line of identity with standard IT-1 polysaccharide at 1 mg/ml. The vaccine did not react with Concanavalin A. A positive control of mannan reacted well with the Concanavalin A, giving a strong precipitin line.

30. The test for immunogenicity of the vaccine was performed in five C3H/HeJ mice. The mice were anesthetized with ether and bled from the retroorbital plexus. They were then given 50 ug of Type 3, Lot 1, vaccine in 0.5 ml sterile saline via an intraperitoneal injection on 8/4/82. Seven days later on 8/11/82 the mice were again anesthetized and bled from the retro-orbital plexus. The serum was allowed to clot at 37° C for 1/2 hour and overnight at 4° C. A Farr test was set up as follows. 100 ul of the pre- and post-immunization sera were mixed with 50 ul of the Farr antigen, which is an intrinsically ^{14}C-labelled polysaccharide preparation, with a specific activity of 0.6 cpm/ng. The antigen and antibody were incubated at 37° C for 2 hours, then placed at 4° C in tightly stoppered tubes over the rest of the weekend. On 8/11/82 the Farr test was completed by adding 150 ul of saturated ammonium sulfate to each of the test tubes, placing the tubes at 4° C for 1/2 hour, followed by addition of 0.5 ml of 50% ammonium sulfate. The precipitate was spun out, washed once in 0.5 ml of 50% ammonium sulfate, the wash poured off, the pellet allowed to dry, then dissolved in Protosol and liquiflor/toluene for counting in a scintillation counter. The results were obtained on 8/11/82 and are as presented in Appendix D. In this experiment only 2 of 5 mice made a measureable immune response.

31. Chemical analyses of the product are presented in Appendix E. The protein and nucleic acid contents were determined in a portion of the material not sent to the State Health Laboratory, because the presence of merthiolate in the final product interfers with these assays. These data indicate that the resultant antigen was composed principally of carbohydrate (about 72%) with low levels of contaminating nucleic acid and protein.

32. Analysis of the coagulation activity of the vaccine in the limulus amebocyte lysate assay indicated that it took 1,000 times more vaccine than LPS from IT-3 P. aeruginosa to gel the limulus amebocyte assay, (and 500,000 times as much vaccine as E. coli LPS, Appendix G).

33. Description of final product - This is a freeze dried preparation of purified high molecular weight polysaccharide vaccine to P. aeruginosa IT-3, prepared from 4 lots of material derived from Strain PBBH 12136-80, a clinical isolate identified as an immunotype 3 strain according to the scheme of Fisher Devlin and Gnabski. Each vial contains 250 ug of the purified polysaccharide and sufficient merthiolate (Thimersol) to have a concentration of 1:30,000 when the product is rehydrated with 2 ml (100 ug/ml) of sterile saline.

Gerald B. Pier, Ph.D.
Instructor in Medicine
Harvard Medical School

APPENDIX D

Notices submitted to the Food and Drug Administration for BB-IND-1495.

Brigham and Women's Hospital
A Teaching Affiliate of Harvard Medical School

Harvard Medical School
Department of Medicine

PLEASE REPLY TO:
CHANNING LABORATORY
180 LONGWOOD AVENUE
BOSTON, MASSACHUSETTS 02115
(617) 732-

August 16, 1982

Harry M. Meyer, Jr., M.D.
Director
Bureau of Biologics
Food and Drug Administration
8800 Rockville Pike
Bethesda, Maryland 20205

Dear Dr. Meyer:

The sponsor, Gerald B. Pier, submits this notice of amendment to BB-IND-1495, for claimed investigational exemption of a new drug under the provisions of section 505-(1) of the Federal Food, Drug and Cosmetic Act and 312.1 of Title 21 of the Code of Federal Regulations. This amendment is a revision of our amendment for Production of a Polysaccharide Vaccine to Pseudomonas aeruginosa and includes the following:

1. Revised protocol for the production of polysaccharide vaccine to serotype 1 (Type 1) polysaccharide vaccine.

2. Revised protocol for the production of vaccine from serotype 2 (Type 2) polysaccharide vaccine to Pseudomonas aeruginosa.

3. Amendment for serotype 3 (Type 3) polysaccharide vaccine to Pseudomonas aeruginosa.

Sincerely,

Gerald B Pier

Gerald B. Pier, Ph.D.
Instructor in Medicine
Harvard Medical School

Brigham and Women's Hospital
A Teaching Affiliate of Harvard Medical School

Harvard Medical School
Department of Medicine

PLEASE REPLY TO:
CHANNING LABORATORY
180 LONGWOOD AVENUE
BOSTON, MASSACHUSETTS 02115
(617) 732-

Information submitted herewith has been noted and filed with your BB-IND 1495. If we have further comments, we will notify you.
J P Salewski 7-14-82
IND Branch, BB, FDA-Date

1495

May 19, 1982

Sam T. Gibson, MD
Director
Division of Biologics Evaluation
Bureau of Biologics
Department of Health and Human Services
Food and Drug Administration
Bethesda, MD 20205

Dear Dr. Gibson:

In response to your letter of April 26, 1982 requesting information for my BB IND 1495, I am enclosing a summary of the data which indicates that the activity of our polysaccharide vaccine for Pseudomonas aeruginosa Type I, was maintained over the storage period.

However, I would like to inform you at this point that this lot of vaccine is no longer currently being used. We have discovered that the mannose component of this vaccine is actually due to a mannan contaminant from the Trypticase Soy Broth media used to grow the organism. Our initial investigation into the presence of media contaminants in our vaccines did not discover this mannan component because of its low presence (approximately 0.03 mg/ml) in the media. Furthermore, our initial investigations indicated that by passing the TSB media through ultra-filtration membranes that remove high molecular weight media components, we reduced our bacterial growth and polysaccharide yield by about 50%. The high molecular weight media components we did find during these initial investigations did not appear to be polysaccharide components. From these data we concluded that media components were not a problem in our polysaccharide preparation.

However, during studies regarding structural configurations of the monosaccharide components in the P. aeruginosa polysaccharides, we deduced that a large portion of the vaccine was in fact a mannan structure. Although the mannan could have been produced by the P. aeruginosa cells, further investigations into its structure indicated that it was very similar to mannan structures produced by strains of yeast. Although there is no yeast extract used in TSB, we decided that the media might be a source of this mannan component. Investigations indicate that there is a component in TSB that strongly reacts with the lectin concanavalin (Con A). Passage of our polysaccharide vaccines over Con A-Sepharose columns eliminated the mannan component from the polysaccharide preparations, whilst the serologically active and immunogenic component passed through the Con A-Sepharose column.

Even after these manipulations were performed, we still thought the mannan component might be essential to our polysaccharide preparations, inasmuch as mannan depleted polysaccharide appeared to have lost its immunogenicity in animals. However, loss of immunogenicity was associated with manipulations of the polysaccharide during the Con A-Sepharose purification procedure, and we have since been able to remove the mannan component from the P. aeruginosa polysaccharide vaccines and maintain their serologic activity and immunogenicity in animals.

Because of these discoveries, we will no longer be using previously prepared P. aeruginosa polysaccharides Types I and II in humans. We are currently preparing polysaccharide vaccines from these two strains of P. aeruginosa, and are incorporating two new steps to eliminate the mannan component. The first step is the passage of the TSB media through ultrafiltration membranes with a molecular weight cutoff of 30,000, in order to remove the high molecular weight mannan components. However, we found this does not eliminate all the detectable mannan components, and have added a Con A-Sepharose chromatography step to insure that all of the mannan components from the media are removed. Once we have completed testing of these new lots of P. aeruginosa polysaccharides, we will submit an amended notice of claimed Investigational exemption for your review.

If you would like any further information or data concerning the information in this letter, please do not hesitate to contact me. I feel that the data regarding the maintenance of activity during the storage period will also apply to these future polysaccharide vaccines without mannan. The mannan component does not appear necessary for immunogenicity and/or serological activity of polysaccharide, nor does it appear that there was any immune response to the mannan component in persons who received the Type I polysaccharide vaccine. These tests involved immunodiffusion against purified mannan. We are currently testing these sera in an ElISA utilizing yeast mannans, to see if any antibody rises to the mannan component occured following immunization with our vaccines.

Sincerely,

Gerald B. Pier, PhD

Instructor in Medicine
HARVARD MEDICAL SCHOOL

bhc
ENC: data sheet

P. aeruginosa Polysaccharide Type I, Lot 2

Dates Prepared:

Start 1/16/81
Finish 2/19/81

Test of Stability of Serologic Activity in Ouchterlony Immunodiffusion

On 2/7/82 polysaccharide Type I, Lot 2 at 1 mg/ml gave a line of identity with P. aeruginosa polysaccharide Type 1, Lot VL-12, (1 mg/ml) completed 12/28/82 and Type 1, Lot VL-9 (1 mg/ml) completed 2/4/81.

Test of Stability of Immunogenicity of Type 1, Lot 2 Polysaccharide

Binding Antibody (ug/ml) in C3H Mice

Date of Immunization	Amount (ug)	Pre-immune	Post-immune
4/17/81	5	3.8	44.9
2/03/82	5	3.9	41.3

APPENDIX E

Documentation of approval from the Brigham and Women's Hospital Human Use Committee for vaccine studies.

Brigham and Women's Hospital
A Teaching Affiliate of Harvard Medical School
10 Vining Street, Boston, Massachusetts 02115
(617) 732-5740

REPORT OF ACTION OF THE COMMITTEE ON HUMAN SUBJECTS

HUMAN SUBJECTS DOCKET NUMBER 5109 PRINCIPAL INVESTIGATOR Gerald B. Pier, Ph.D.

TITLE: Safety and Immunogenicity Testing of a Pilot Polysaccharide Vaccine Preparation to Pseudomonas Aeruginosa

This is to certify that the application identified above has been reviewed by the Committee appointed to review proposals involving clinical research and other investigations involving human beings, which has considered specifically:

(1) the rights and welfare of the individual or individuals involved,
(2) the appropriateness of the methods used to secure informed consent, and
(3) the risks and potential medical benefits of the investigation.

The Human Subjects Committee reviewed your research protocol and recommend approval. Please use enclosed authorized copy of consent form and/or questionnaires in your research.

NOTE: Approvals are granted for the period of one year only and must be renewed annually. In addition, adverse reactions of any kind must be reported immediately in writing to the Committee, as they occur.

FOR THE HUMAN SUBJECTS COMMITTEE

9/14/81
DATE OF COMMITTEE ACTION

Dr. Robert Handin
CHAIRMAN

ADM. APP.
ASSIGNED TO GROUP

E.L. Mayhew
EXECUTIVE SECRETARY

FOR PROTOCOL C.R. #4899

RESEARCH CONSENT FORM

DATE PREPARED: August 12, 1981

PROJECT TITLE: ~~Safety and Immunogenicity Testing~~ of a Pilot Polysaccharide Vaccine Preparation to Pseudomonas aeruginosa.

PHYSICIAN(s): _____

IDENTIFYING NUMBER(s) _____

VOLUNTEER/PATIENT NAME: _____
If not imprinted above)

EV. 9/80

APPROVED FOR USE BY THE BRIGHAM AND WOMEN'S HOSPITAL
9-14-81

SIGNED BY: B.L. Mayhew
Secretary, Human Subjects Committ

DOCKET NUMBER: 5109

EXPIRATION DATE: 9-13-82

HEALTHY VOLUNTEER CONSENT FORM

You understand that you will be given an injection in your arm of a possible vaccine to a bacterial infection.

This bacterial infection, called Pseudomonas aeruginosa infection, is seen only in patients in hospitals whose resistance is lowered because of some physical or disease problem that they have. The infection is most often seen as a disease when these bacteria get into either the blood or lungs, causing a blood sepsis or lung pneumonia. About 60% of the time these conditions are fatal.

You understand that the vaccine is composed of a polysaccharide material, the composition of which has been explained to you. You may see that your arm will show a red area and be sore and tender for up to 3 days. Shortly after you receive the injection, although it is highly unlikely, there is a very slight possibility of a more severe reaction, called anaphylaxis, that would cause difficulty in breathing, an ashen color, a drop in blood pressur and possibly an irregular heartbeat. Precautionary measures have been taken to aid you in the event of a bad reaction. This treatment would involve injection of 1-10 cc of epinephrine into a muscle, a treatment known to reverse the anaphylaxis reaction. You understand that about 1 ounce of blood will be taken from your arm in 1 minute prior to receiving the injecti This blood sampling procedure may result in a slight hematoma, commonly known as a black and blue mark. You understand that about 1 ounce of blood will be taken in 1 minute from your arm 14, 28, 180 and 365 days after getting the injection. You will be asked questions about how you feel after getting the injection and you are to report any reactions you think may be associated with the injection. Your blood samples will be analyzed for your body's reaction t the injection. You understand that the blood information derived from your participation in this study will be tabulated and analyzed. The blood tests and specimen collection are routin procedures and the tabulations and analysis are research procedures. You will not be identified in any publications of this data.

You understand that you do not have to take part in this study if you do not want to. You understand that you can refuse any or all of the procedures and that you are free to withdraw from this study at any time. Your questions have been answered to your satisfaction and you understand that Joan Biggers, the patient representative will be available to answer any questions that may arise during the course of this study. It has been explained to you that you can expect little to no personal benefit from participating in this test unless, by a coincidence, the vaccine works and in the future you become infected with the bacteria to which this vaccine is directed against.

You will be paid $10 for your initial participation in donating a blood sample and for receiving the vaccine. You will be paid $5 for your subsequent blood donations.

Continued.....

You agree to participate in this study which as been explained to you in detail.

Subject:_____ Date:_____

I have explained the above to the subject.

Investigator:_____Date:_____

I attest that the contents of this form have been adequately and appropriately explained to the subject.

Witness:_____Date:_____

RESEARCH CONSENT FORM

DATE PREPARED: August 12, 1981

PROJECT TITLE: Safety and Immunogenicity Testing of a Pilot Polysaccharide Vaccine Preparation to Pseudomonas aeruginosa.

PHYSICIAN(s):

IDENTIFYING NUMBER(s)

VOLUNTEER/PATIENT NAME:
If not imprinted above)

EV. 9/80

APPROVED FOR USE BY THE BRIGHAM AND WOMEN'S HOSPITAL
9-14-81

SIGNED BY: B.L. Mayhew
Secretary, Human Subjects Commi

DOCKET NUMBER: 5109

EXPIRATION DATE: 9-13-82

HOSPITALIZED PATIENT CONSENT FORM

You understand that you will be given an injection in your arm of a possible vaccine to a bacterial infection or simply a shot of saline (salt water).

This bacterial infection, called Pseudomonas aeruginosa infection, is seen only in patients in hospitals whose resistance is lowered because of some physical or disease problem that they have. The infection is most often seen as a disease when these bacteria get into either the blood or lungs, causing a blood sepsis or lung pneumonia. About 60% of the time these conditions are fatal.

You understand that the vaccine is composed of a polysaccharide material, the composition of which has been explained to you. You may see that your arm will show a red area and be sore and tender for up to 3 days. Shortly after you receive the injection, although it is highly unlikely, there is a very slight possibility of a more severe reaction, called anaphylaxis, that would cause difficulty in breathing, an ashen color, a drop in blood pressure and possibly an irregular heartbeat. Precautionary measures have been taken to aid you in the event of a bad reaction. This treatment would involve injection of 1-10 cc of epinephrine into a muscle, a treatment known to reverse the anaphylaxis reaction. You understand that about 1 ounce of blood will be taken from your arm in one minute prior to receiving the injection. This blood sampling procedure may result in a slight hematoma, commonly known as a black and blue mark. You understand that about 1 ounce of blood will be taken in 1 minute from your arm 7, 14 and 28 days after getting the injection. You will be asked questi about how you feel after getting the injection and you are to report any reactions you think may be associated with the injection. Your blood samples will be analyzed for your body's reaction to the injection. You understand that the blood information derived from your participation in this study will be tabulated and analyzed. The blood tests and specimen collection are routine procedures and the tabulations and analysis are research procedures. You will not be identified in any publications of this data.

You understand that there is a one out of four chance that you will not be getting the experimental vaccine but only the material which is used to dissolve the vaccine (saline). This is because in order to test the effectiveness of the vaccine we need a group of people who have not been given the vaccine as a comparison or control group. Since this vaccine is only an experimental one, and not available for use other than in this test, you would not be denied the benefit of any currently available treatments for preventing infections.

You understand that you do not have to take part in this study if you do not want to. You understand that you can refuse any or all of the procedures and that you are free to withdraw from this study at any time. You understand that your agreement or refusal to participate in this study will in no way affect you medical care. Your questions have been answered to you satisfaction and you understand that Joan Biggers, the patient representative will be avail to answer any questions that may arise during the course of this study.

Continued....

It has been explained to you that you can expect little to no personal benefit from participating in this test unless, by a coincidence, the vaccine works and in the future you become infected with the bacteria to which this vaccine is directed against.

You agree to participate in this study which has been explained to you in detail.

Subject:_____ Date:_____

I have [illegible]

Investigator:_____ Date:_____

I attest that the contents of this form have been adequately and appropriately explained to the subject.

Witness:_____ Date:_____

DEPARTMENT OF HEALTH AND HUMAN SERVICES

PROTECTION OF HUMAN SUBJECTS ASSURANCE/CERTIFICATION/DECLARATION

☐ ORIGINAL ☐ FOLLOWUP ☐ REVISION

☐ GRANT [XX] CONTRACT ☐ FELLOW ☐ OTHER

☐ NEW [X] RENEWAL ☐ CONTINUATION

APPLICATION IDENTIFICATION NUMBER *(If known)*
DAMD-17-79C-9050

STATEMENT OF POLICY: Safeguarding the rights and welfare of subjects at risk in activities supported under grants and contracts from DHHS is primarily the responsibility of the institution which receives or is accountable to DHHS for the funds awarded for the support of the activity. In order to provide for the adequate discharge of this institutional responsibility, it is the policy of DHHS that no activity involving human subjects to be supported by DHHS grants or contracts shall be undertaken unless the Institutional Review Board has reviewed and approved such activity, and the institution has submitted to DHHS a certification of such review and approval, in accordance with the requirements of Public Law 93-348, as implemented by Part 46 of Title 45 of the Code of Federal Regulations, as amended, (45 CFR 46). Administration of the DHHS policy and regulation is the responsibility of the Office for Protection from Research Risks, National Institutes of Health, Bethesda, MD 20205.

1. TITLE OF PROPOSAL OR ACTIVITY

SAFETY AND IMMUNOGENICITY TESTING OF A PILOT POLYSACCHARIDE VACCINE PREPARATION TO PSEUDOMONAS AERUGINOSA

2. PRINCIPAL INVESTIGATOR/ACTIVITY DIRECTOR/FELLOW

GERALD B. PIER, PH.D.

3. DECLARATION THAT HUMAN SUBJECTS EITHER WOULD OR WOULD NOT BE INVOLVED

☐ A. NO INDIVIDUALS WHO MIGHT BE CONSIDERED HUMAN SUBJECTS, INCLUDING THOSE FROM WHOM ORGANS, TISSUES, FLUIDS, OR OTHER MATERIALS WOULD BE DERIVED, OR WHO COULD BE IDENTIFIED BY PERSONAL DATA, WOULD BE INVOLVED IN THE PROPOSED ACTIVITY. (IF NO HUMAN SUBJECTS WOULD BE INVOLVED, CHECK THIS BOX AND PROCEED TO ITEM 7. PROPOSALS DETERMINED BY THE AGENCY TO INVOLVE HUMAN SUBJECTS WILL BE RETURNED.)

[X] B. HUMAN SUBJECTS WOULD BE INVOLVED IN THE PROPOSED ACTIVITY AS EITHER: [X] NONE OF THE FOLLOWING, OR INCLUDING: ☐ MINORS, ☐ FETUSES, ☐ ABORTUSES, ☐ PREGNANT WOMEN, ☐ PRISONERS, ☐ MENTALLY RETARDED, ☐ MENTALLY DISABLED. UNDER SECTION 6. COOPERATING INSTITUTIONS, ON REVERSE OF THIS FORM, GIVE NAME OF INSTITUTION AND NAME AND ADDRESS OF OFFICIAL(S) AUTHORIZING ACCESS TO ANY SUBJECTS IN FACILITIES NOT UNDER DIRECT CONTROL OF THE APPLICANT OR OFFERING INSTITUTION.

4. DECLARATION OF ASSURANCE STATUS/CERTIFICATION OF REVIEW

☐ A. THIS INSTITUTION HAS NOT PREVIOUSLY FILED AN ASSURANCE AND ASSURANCE IMPLEMENTING PROCEDURES FOR THE PROTECTION OF HUMAN SUBJECTS WITH THE DHHS THAT APPLIES TO THIS APPLICATION OR ACTIVITY. ASSURANCE IS HEREBY GIVEN THAT THIS INSTITUTION WILL COMPLY WITH REQUIREMENTS OF *DHHS Regulation 45 CFR 46*, THAT IT HAS ESTABLISHED AN INSTITUTIONAL REVIEW BOARD FOR THE PROTECTION OF HUMAN SUBJECTS AND, WHEN REQUESTED, WILL SUBMIT TO DHHS DOCUMENTATION AND CERTIFICATION OF SUCH REVIEWS AND PROCEDURES AS MAY BE REQUIRED FOR IMPLEMENTATION OF THIS ASSURANCE FOR THE PROPOSED PROJECT OR ACTIVITY.

[X] B. THIS INSTITUTION HAS AN APPROVED GENERAL ASSURANCE (DHHS ASSURANCE NUMBER M1049-01) OR AN ACTIVE SPECIAL ASSURANCE FOR THIS ONGOING ACTIVITY, ON FILE WITH DHHS. THE SIGNER CERTIFIES THAT ALL ACTIVITIES IN THIS APPLICATION PROPOSING TO INVOLVE HUMAN SUBJECTS HAVE BEEN REVIEWED AND APPROVED BY THIS INSTITUTION'S INSTITUTIONAL REVIEW BOARD IN A CONVENED MEETING ON THE DATE OF 9/14/81 IN ACCORDANCE WITH THE REQUIREMENTS OF THE *Code of Federal Regulations on Protection of Human Subjects (45 CFR 46)*. THIS CERTIFICATION INCLUDES, WHEN APPLICABLE, REQUIREMENTS FOR CERTIFYING FDA STATUS FOR EACH INVESTIGATIONAL NEW DRUG TO BE USED (SEE REVERSE SIDE OF THIS FORM).

THE INSTITUTIONAL REVIEW BOARD HAS DETERMINED, AND THE INSTITUTIONAL OFFICIAL SIGNING BELOW CONCURS THAT:

EITHER ☐ HUMAN SUBJECTS WILL NOT BE AT RISK; OR [X] HUMAN SUBJECTS WILL BE AT RISK.

5. AND 6. SEE REVERSE SIDE

7. NAME AND ADDRESS OF INSTITUTION

Peter Bent Brigham Hospital, A Division of Brigham and Women's Hospital
10 Vining Street
Boston, MA 02115

8. TITLE OF INSTITUTIONAL OFFICIAL	TELEPHONE NUMBER
Burton L. Mayblum, Secretary Human Subjects Committee	732-5740
SIGNATURE OF INSTITUTIONAL OFFICIAL	DATE
B L Mayblum	August 18, 1982

HHS-596 (Rev. 5-80)

ENCLOSE THIS FORM WITH THE PROPOSAL OR RETURN IT TO REQUESTING AGENCY.

5. INVESTIGATIONAL NEW DRUGS - ADDITIONAL CERTIFICATION REQUIREMENT

SECTION 46.17 OF TITLE 45 OF THE Code of Federal Regulations states, "Where an organization is required to prepare or to submit a certification . . . and the proposal involves an investigational new drug within the meaning of The Food, Drug, and Cosmetic Act, the drug shall be identified in the certification together with a statement that the 30-day delay required by 21 CFR 130.3(a)(2) has elapsed and the Food and Drug Administration has not, prior to expiration of such 30-day interval, requested that the sponsor continue to withhold or to restrict use of the drug in human subjects; or that the Food and Drug Administration has waived the 30-day delay requirement; provided, however, that in those cases in which the 30-day delay interval has neither expired nor been waived, a statement shall be forwarded to DHHS upon such expiration or upon receipt of a waiver. No certification shall be considered acceptable until such statement has been received."

INVESTIGATIONAL NEW DRUG CERTIFICATION

TO CERTIFY COMPLIANCE WITH FDA REQUIREMENTS FOR PROPOSED USE OF INVESTIGATIONAL NEW DRUGS IN ADDITION TO CERTIFICATION OF INSTITUTIONAL REVIEW BOARD APPROVAL, THE FOLLOWING REPORT FORMAT SHOULD BE USED FOR EACH IND: (ATTACH ADDITIONAL IND CERTIFICATIONS AS NECESSARY).

— IND FORMS FILED: [X] FDA 1571, [X] FDA 1572, [ ] FDA 1573

— NAME OF IND AND SPONSOR Pseudomonas aeruginosa polysaccharides- Types 1 and 2
Gerald B. Pier, sponser

— DATE OF 30-DAY EXPIRATION OR FDA WAIVER
(FUTURE DATE REQUIRES FOLLOWUP REPORT TO AGENCY) September 20, 1982

— FDA RESTRICTION _____

— SIGNATURE OF INVESTIGATOR Gerald B Pier DATE 8/20/82

6. COOPERATING INSTITUTIONS - ADDITIONAL REPORTING REQUIREMENT

SECTION 46.16 OF TITLE 45 OF THE *Code of Federal Regulations* IMPOSES SPECIAL REQUIREMENTS ON THE CONDUCT OF STUDIES OR ACTIVITIES IN WHICH THE GRANTEE OR PRIME CONTRACTOR OBTAINS ACCESS TO ALL OR SOME OF THE SUBJECTS THROUGH COOPERATING INSTITUTIONS NOT UNDER ITS CONTROL. IN ORDER THAT THE DHHS BE FULLY INFORMED, THE FOLLOWING REPORT IS REQUESTED WHEN APPLICABLE.

USE FOLLOWING REPORT FORMAT FOR EACH INSTITUTION OTHER THAN GRANTEE OR CONTRACTING INSTITUTION WITH RESPONSIBILITY FOR HUMAN SUBJECTS PARTICIPATING IN THIS ACTIVITY: (ATTACH ADDITIONAL REPORT SHEETS AS NECESSARY).

INSTITUTIONAL AUTHORIZATION FOR ACCESS TO SUBJECTS

— SUBJECTS: STATUS (WARDS, RESIDENTS, EMPLOYEES, PATIENTS, ETC.) _____

NUMBER _____ AGE RANGE _____

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NOTES: *(e.g., report of modification in proposal as submitted to agency affecting human subjects involvement)*

H. Copies of manuscripts not previously submitted to the USAMRDC.

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INDUCTION IN MICE OF CELL-MEDIATED IMMUNITY TO *PSEUDOMONAS AERUGINOSA* BY HIGH MOLECULAR WEIGHT POLYSACCHARIDE AND VINBLASTINE[1]

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The effect of the cytotoxic drug vinblastine on the development of immunity to high m.w. polysaccharide (PS) isolated from culture supernates of *Pseudomonas aeruginosa* was investigated. One microgram of PS, a normally nonimmunogenic, nonprotective dose, plus 75 μg of vinblastine were administered to BALB/c mice, and afforded protection to live organism challenge with the homologous strain. The kinetics and serotype specificity of the immune response indicated an active immunization had occurred. Analyses of serum antibody levels of mice given the PS-drug regimen in a sensitive, radioactive antigen-binding assay (RABA) failed to show development of antibody to the immunizing PS. Immunity could be passively transferred with spleen cells but not by serum from PS-drug-immunized animals, and the effector cell was removed by antisera to the Thy-1.2 antigen. Nu/nu mice were also protected against challenge after immunization with PS and vinblastine, but this protection was observed in association with the development of serum antibody to PS in these mice, as measured in the RABA. Protective immunity could not be elicited in the BALB/c mice by PS plus cyclophosphamide. These data suggest that under certain conditions, PS antigens can elicit T cell-dependent immune phenomena, and this T cell-dependent immunity can protect mice from live organism challenge against an extracellular bacterial pathogen.

Immune protection against extracellular bacterial pathogens has been well correlated with the development of antibodies specific for antigens present on bacterial surfaces (1). The ability of antibodies to promote phagocytosis and killing of extracellular bacteria *in vitro* (2) and the protection afforded to nonimmune recipients by passively transferred immune sera (3) demonstrated the critical role antibodies play in immunity against such organisms. It has also been amply documented that killing of those bacteria, such as *Listeria monocytogenes*, that live intracellularly and are inaccessible to the effects of antibodies is critically dependent on the function of effector T cells (4). Antibody provides minimal if any protection against such organisms (2, 4).

The possibility that T cells might also play a role in immunity to extracellular bacteria has not been extensively explored. Difficulty in demonstrating a role for effector T cells in situations in which antibody-mediated protection is so efficient, probably accounts, in part, for the paucity of data on the role of cell-mediated immunity (CMI)[3] in relation to extracellular bacterial infections. Recent studies of protection induced in mice by immunization with a high m.w. polysaccharide (PS) antigen isolated from culture supernates of the extracellular bacterial pathogen, *Pseudomonas aeruginosa* immunotype (IT-1) (5), demonstrated the ability of this PS to induce antibodies that protect mice from live bacterial challenge (6, 7). During the course of these protection studies, it was discovered that BALB/c mice generated a protective antibody response to a 50-μg dose of the IT-1 PS, but failed to produce antibodies after immunization with a limiting 1 μg dose of IT-1 PS. Mice were also not protected from subsequent live bacterial challenge after the low dose immunization. It was observed, however, that if BALB/c mice received the mitotic inhibitor vinblastine sulfate, at the time of immunization with 1 μg of the IT-1 PS, they were protected against bacterial challenge, despite the fact they still failed to produce an antibody response. The present report provides evidence that the protection against *P. aeruginosa* infection observed in BALB/c mice receiving vinblastine plus 1 μg of IT-1 PS is T cell-mediated.

MATERIALS AND METHODS

Immunogens. High m.w. PS from *P. aeruginosa* IT-1 was prepared as previously described (5). Vinblastine sulfate (Velban) was obtained from Eli Lilly and Co., Indianapolis, IN. Cyclophosphamide (Cytoxan) was obtained from Meade Johnson Pharmaceutical Co., Evansville, IN.

Bacteria. Strains of *P. aeruginosa* IT 1–7 were kindly provided by Dr. M. Fisher, Parke-Davis Co., Detroit, MI.

Mice. Eight-week-old BALB/c mice were obtained from Cumberland View Farms, Clinton, TN. Nu/nu and Nu/+ littermate BALB/c mice were obtained from Charles River Breeders, Wilmington, MA.

Immunization and challenge protocol. Mice were immunized with the indicated dosage of IT-1 PS in 0.5 ml of phosphate-buffered saline (PBS, pH 7.2). The PBS used had to be passed through a DM-5 ultrafiltration membrane (Amicon Corp., Danvers, MA) to eliminate a factor that gave nonspecific protection with vinblastine alone. The vinblastine was given as a 75-μg dose in 0.2 ml ultrafiltered PBS. Cyclophosphamide was administered in 0.2 ml ultrafiltered PBS i.v. 48 hr before antigen. The mice were challenged at the indicated time interval after immunization with live *P. aeruginosa* organisms in 0.1 ml saline i.p. The challenge dose for *P. aeruginosa* IT-1, 2, and 4 was 10^6 organisms per mouse; for *P. aeruginosa* IT-5, 6, and 7, 10^7 organisms per mouse; and for *P. aeruginosa* IT-3, 5×10^6 organisms per mouse. The challenge doses were prepared from overnight cultures grown on trypticase soy agar, suspended in saline, standardized by optical density measurements at 650 nm, then administered as a 0.1 ml i.p. dose. Deaths were recorded for 96 hr after challenge.

Antisera. Murine sera were obtained via retroorbital bleeding of ether-anesthetized animals.

Serologic assay. Concentrations of antibody in micrograms per milliliter were determined by use of a radioactive antigen-binding assay (RABA) as

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[3] Abbreviations used in this paper: PS, polysaccharide; IT, immunotype; RABA, radioactive antigen-binding assay; CMI, cell-mediated immunity; HEPES, *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid; MIF, macrophage inhibition factor.

described (7, 8). The antigen used was intrinsically labeled ^{14}C PS prepared as described (7, 8). The lower limit of detection of antibody in this assay for mouse antibodies is 2 μg/ml.

Passive transfer studies. Passive transfer of serum was done by giving the indicated amount of mouse antiserum i.p. 3 hr before challenge with 10^6 live *P. aeruginosa* IT-1 organisms. Passive transfer of spleen cells was performed by transferring the indicated number of cells in 0.5 ml Hanks' balanced salt solution (HBSS, Microbiological Associates, Bethesda, MD) via an i.p. injection. Cells were obtained from the spleens of animals by passing the intact spleens through a wire mesh screen to disperse the cells, then removing large fragments by passing the cell suspension through a funnel loosely packed with glass wool. The cells in this suspension were pelleted by centrifugation and resuspended in HBSS, viable cell counts were determined by trypan blue dye exclusion, and the cell concentration was adjusted to the desired amount. Challenge of recipient mice was 72 hr later with 10^6 live *P. aeruginosa* IT-1 cells.

Selection of lymphocyte subpopulations. T cells and B cells were selectively removed from spleen cell suspensions by incubation of 3×10^7 cells in 3 ml HBSS on petri plates coated with either mouse anti-Thy-1.2 sera, rabbit antisera to mouse immunoglobulin (Ig, Accurate Chemical Co., Hicksville, NY), or normal mouse serum (NMS) for 45 min at 4°C (9). The nonadherent cells were removed, were pelleted by centrifugation, and were resuspended in the volume of HBSS that gave 2×10^7 cells before the depletion step.

Determination of efficacy of lymphocyte subpopulation separations. After depletion of either T cells or B cells, an aliquot of the cell suspension was taken and the cells were pelleted by centrifugation and resuspended in RPMI 1640 medium containing HEPES[4] buffer, L-glutamine, penicillin, and streptomycin. The volume was chosen to give 10^7 cells/ml before depletion. Then, 100 μl (10^6 cells) of this cell suspension were placed into round-bottomed microtiter wells and the following mitogens were added to triplicate cultures: phytohemagglutinin (PHA), 2 μg/ml; concanavalin A (Con A), 0.2 μg/ml; and lipopolysaccharide (LPS), 5.0 μg/ml, from *Escherichia coli* 0:26 (Difco Laboratories, Detroit, MI). Cultures were then assayed for mitogenic stimulation as described (8).

Statistics. P values for survival from live cell challenge were calculated by a Fisher Exact Test. P values for differences in pre- and post-immunization serum antibody levels were calculated by a *t*-test.

RESULTS

Protection of BALB/c mice by immunization with IT-1 PS plus vinblastine. Groups of 10 mice were immunized with either 1 μg of IT-1 PS plus 75 μg vinblastine, 1 μg of IT-1 PS alone, 75 μg of vinblastine alone, or ultrafiltered PBS, and were challenged 7 days later with 10^6 live *P. aeruginosa* IT-1 cells i.p. The data (Table I) show that the mice given 1 μg IT-1 PS i.p. plus 75 μg vinblastine i.v. (within 5 min of each other) were significantly ($p < 0.001$, Fisher Exact Test) protected from challenge with live *P. aeruginosa* IT-1 cells. No protection was evident among mice given the PS or drug alone, or mice given PBS.

Immunotype specificity of PS plus vinblastine-induced protection. To determine if the protection observed was specific, four groups of 70 mice each were immunized with either 1 μg of the IT-1 PS plus 75 μg of vinblastine, 1 μg of the IT-1 PS only, 75 μg of vinblastine only, or PBS. Within each group of 70, subgroups of 10 mice were challenged 7 days later with the indicated doses of live *P. aeruginosa* cells from each of the seven Fisher immunotypes. Protection was only seen against challenge with the homologous IT-1 strain of *P. aeruginosa* in the mice immunized with 1 μg IT-1 PS plus 75 μg of vinblastine (Table II), indicating the serotype specificity of the immunity elicited.

Kinetics of induction of protective immunity. Kinetics of the immunity induced by immunization with the PS-drug protocol was then examined. Five days after immunization, 40% protection was noted (Table III), which increased to 100% by the sixth day after immunization. The protective level fell to 40% by day 17 post-immunization. Attempts to boost the duration of immune protection by giving three doses of the PS-drug regimen at 7-day intervals indicated 80 to 100% protection 35 days after the last injection (Table IV). The immunity began to wane after this time, but significant protection was still observed 49 days after the final dose (Table IV).

The effect of varying the time interval between PS immunization and vinblastine administration was next studied. Vinblastine given from 72 hr before until 8 hr after PS was effective in eliciting protection from live organism challenge (Table V).

TABLE I

Protection of BALB/c mice to live organism challenge after immunization with high m.w. PS from P. aeruginosa IT-1 and vinblastine

Immunized with:	Dose	No. Mice per Group	Number Survivors	Percentage Survivors	P Value
	μg				
High m.w. PS plus Vinblastine	1 75	10[a]	8	80	<0.001
High m.w. PS	1	10	0	0	
Vinblastine	75	10	2	20	0.24
PBS		10	0	0	

[a] These mice bled for serum antibody studies in Table VI.

TABLE II

Serotype specificity of protection elicited in BALB/c mice given 1μg IT-1 high m.w. PS plus 75 μg vinblastine

Serotype of Challenge Strain	PS[a] + Vin	Vin Only	PS Only	PBS Only	P Value PS + Vin vs PBS only
IT-1	10^b/10	0/10	1/10	0/10	<0.001
IT-2	2/10	1/10	0/10	0/10	0.24
IT-3	2/10	2/10	1/10	0/10	0.24
IT-4	0/10	1/10	1/10	0/10	1.0
IT-5	0/10	0/10	0/10	0/10	1.0
IT-6	0/10	0/10	1/10	0/10	1.0
IT-7	1/10	0/10	0/10	0/10	0.5

[a] PS, high m.w. polysaccharide from IT-1 *P. aeruginosa*; Vin, vinblastine.
[b] Represents number survivors over total challenged. Mice immunized with PS + Vin and challenged with IT-1 cells were bled for serum antibody study in Table VI.

TABLE III

Kinetics of protection of BALB/c mice after immunization with 1 μg IT-1 high m.w. PS and 75 μg vinblastine

Days between immunization and Challenge	Immunized with: PS[a] + VIN	VIN only	PS only	PBS only	P Value PS+Vin vs PBS
24	3^b/10	1/10	0/10	0/10	0.105
21	5/10	1/10	0/10	0/10	0.016
17	4/10	0/10	0/10	0/10	0.043
14	8/10	0/10	1/10	0/10	<0.001
10	9/10	0/10	1/10	0/10	<0.001
7	10^c/10	1/10	1/10	0/10	<0.001
6	10/10	1/10	0/10	0/10	<0.001
5	4/10	0/10	1/10	0/10	0.043
4	0/10	0/10	0/10	0/10	1.0
3	0/10	1/10	1/10	0/10	1.0
2	0/10	0/10	0/10	0/10	1.0
1	0/10	1/10	0/10	0/10	1.0

[a] PS, high m.w. polysaccharide from IT-1 *P. aeruginosa*; Vin, vinblastine.
[b] Number survivors over total challenged.
[c] These mice bled for serum antibody determinations in Table VI.

TABLE IV

Increase in duration of immunity in BALB/c mice given 3 doses of 1 μg IT-1 PS plus 75 μg vinblastine at 7-day intervals

Days after Final Injection	Immunized with: PS + Vin	Vin only	PS only	PBS only	P Value, PS + Vin vs PBS
17	10^a/10	1/10	0/10	0/10	0.001
49	4/10	0/10	1/10	0/10	0.043
45	6/10				
42	6/10				
35	10/10				
31	10/10				
24	8/10				
21	10/10				

[a] Number of survivors over total challenged. Mice challenged 17 days after the final injection were bled for serum antibody determinations in Table VI.

TABLE V

Effect on development of immunity by varying the time interval between administration of 75 μg vinblastine and 1 μg IT-1 high m.w. PS

Interval between Vinblastine and PS Administration	No. Survivors Total challenged	P Value (vs PBS only)
−96	0/10	1.0
−72	10/10	<0.001
−48	10/10	<0.001
−24	10/10	<0.001
0[a]	9/10	<0.001
+2	10/10	<0.001
+4	10/10	<0.001
+8	8/10	<0.001
+24	2/10	0.24
+36	1/10	0.5
Vin only	1/10	0.24
PS only	0/10	1.0
PBS only	0/10	

[a] These mice bled for serum antibody determination in Table VI.

TABLE VI

Serum antibody levels (μg/ml) of mice given 1 μg IT-1 high m.w. PS plus 75 μg vinblastine in experiments in Tables I to V

Expt. in Table:	No. Mice	Preimmune Avg. (range)[a]	Postimmune Avg. (range)	Difference of Avg. (μg/ml)	P Value[b]
I	10	2.8 (<2–4.1)	2.9 (<2–4.0)	0.1	NS[c]
II	10	2.7 (<2–3.9)	2.6 (<2–3.9)	−0.1	NS
III	10	2.8 (<2–3.3)	2.2 (<2–3.3)	−0.6	NS
IV	10	3.1 (<2–4.5)	3.3 (<2–4.6)	0.2	NS
V	10	2.6 (<2–3.9)	2.9 (<2–4.5)	0.3	NS
50 μg PS (control)	10	2.2 (<2–4.1)	25.4 (14.7–30.7)	23.2	<0.01

[a] Lower limit of test sensitivity for mouse serum is 2 μg/ml.
[b] Calculated by Student's *t*-test.
[c] NS, nonsignificant, $p > 0.05$.

Vinblastine given 96 hr before PS immunization, or 24 hr or more after PS, failed to induce protection.

Involvement of serum factors in PS-vinblastine-induced immunity. To understand the basis for the apparent immunity, serum samples taken from some of the mice in the above experiments (Tables I–V) 3 hr before challenge were analyzed for serum antibody to IT-1 PS in the RABA. The groups of mice tested are denoted by footnotes in Tables I–V. Positive controls for this test were sera obtained from BALB/c mice immunized with a 50-μg dose of IT-1 PS, which is known to stimulate antibody formation. Table VI shows that in none of 50 mice given 1 μg IT-1 PS plus 75 μg of vinblastine was there any evidence for an increase in serum antibody levels. Mice immunized with 50 μg of the IT-1 PS alone, however, showed good serum antibody rises.

To ascertain if another serum factor, not measurable in the RABA, was the responsible protective entity, we transferred serum from mice immunized 7 days previously with either 1 μg IT-1 PS alone, 50 μg IT-1 PS alone, 1 μg IT-1 PS plus 75 μg vinblastine, 75 μg vinblastine only, or NMS to nonimmune recipients. Mice were then challenged 3 hr after passive transfer of serum with about 10^6 live *P. aeruginosa* IT-1 cells. Only mice given 0.25 ml of serum from the group immunized with the immunogenic 50-μg dose of IT-1 PS were protected from live organism challenge (Table VII). Up to 0.6 ml of the other sera were not effective in transferring immunity. Above the 0.6-ml serum amount, all of the sera, including NMS, had protective efficacy. This phenomenon has been noted in passive transfer of nonimmune sera to recipient mice challenged with other Gram-negative organisms (10).

Role of immune spleen cells in transferring PS-drug-induced immunity. Because serum factors did not appear to be involved in the immunity elicited by the PS-vinblastine combination, we looked at the ability of spleen cells to transfer immunity. Table VIII shows that 10^7 cells from mice given the PS-drug regimen, when transferred to a recipient mouse, were effective in providing protection to live cell challenge 72 hr after adoptive cell transfer. No passive protection was noted when up to 10^8 cells taken from nonimmune animals or animals given either 1 μg IT-PS alone or 75 μg vinblastine alone, were transferred to recipients. No detectable antibody, as measured in the RABA, was found in the adoptively transferred recipient mice 3 hr before challenge (data not shown).

Identity of the effector cell transferring immunity. Immune spleen cell populations were selectively depleted of lymphocytes bearing either the Thy-1.2 determinant or surface Ig by treatment of 4°C on petri plates coated with either mouse anti-Thy-1.2, rabbit anti-mouse Ig, or NMS (9). The selected populations were then transferred to nonimmune recipient mice that were challenged 72 hr later with live *P. aeruginosa* IT-1 cells. The results (Table IX) indicated that the effector cell transferring immunity was removed by antisera directed at the Thy-1.2 determinant and not by antisera directed at the Ig determinants. Testing of these cell populations for mitogenic stimulation by the T cell mitogens PHA and Con A, and the B cell mitogen, LPS, showed the anti-Thy-1.2-treated cells only

TABLE VII

Passive protection of mice after transfer of mouse serum raised to either 50 μg IT-1 PS, 1 μg IT-1 PS plus 75 μg vinblastine, 1 μg IT-1 PS only, 75 μg vinblastine only or normal mouse sera (NMS)

Serum Given (raised to)	Amt	No. Survivors Total recipients	P Value vs NMS Control
	ml		
50 μg IT-1 PS	0.25	5/5	0.001
1 μg IT-1 PS + 75 μg Vinblastine	0.25	0/5	NS[a]
	0.50	0/5	NS
	0.60	2/5	NS
	0.70	4/5	NS
1 μg IT-1 PS only	0.25	0/5	NS
	0.50	1/5	NS
	0.60	4/5	NS
	0.70	5/5	NS
75 μg Vinblastine only	0.25	0/5	NS
	0.50	1/5	NS
	0.60	3/5	NS
	0.70	4/5	NS
NMS	0.25	0/5	
	0.50	0/5	
	0.60	3/5	
	0.70	5/5	

[a] NS, nonsignificant, $p > 0.5$.

TABLE VIII

Protection of BALB/c mice by adoptive transfer of spleen cells from mice immunized with 1 μg high m.w. IT-1 PS and 75 μg vinblastine (Vin)

Cells Given (raised to)	Amount ($\times 10^7$)	No. Survivors Total recipients	P Value vs Nonimmune Controls
1 μg PS + 75 μg Vin	0.25	0/5	NS[a]
	0.50	1/5	NS
	1.00	5/5	<0.001
	1.25	5/5	<0.001
1 μg PS only	0.50	0/5	NS
	1.00	0/5	NS
	10.00	0/5	NS
75 μg Vin only	0.50	0/5	NS
	1.00	0/5	NS
	10.00	0/5	NS
PBS (nonimmune)	0.50	0/5	
	1.00	1/5	
	10.00	0/5	

[a] NS, nonsignificant, $p > 0.5$.

had 18% of the PHA responsiveness, and 12% of the Con A responsiveness of NMS treated cell populations, while retaining 84% of their LPS responsiveness (Table IX). Anti-Ig-depleted cell populations retained 66% of their PHA and 84% of their Con A response, while the LPS-induced mitogenesis was reduced to 19% of NMS treated controls.

Protection in nu/nu BALB/c mice. We then looked at the ability of 1 μg of IT-1 PS plus 75 μg of vinblastine to protect nu/nu and nu/+ littermate control mice from live organism challenge 7 days after immunization. As expected, neither nu/nu or nu/+ mice were protected after a dose of 1 μg IT-1 PS alone or 75 μg of vinblastine alone (Table X). Unexpectedly, nu/nu mice were protected from live cell challenge after 1 μg of IT-1 PS plus 75 μg of vinblastine, as were the control nu/+ mice. However, when serum antibody levels (difference in post and preimmunization levels) of these mice were measured in the RABA, the nu/nu mice that received the PS-drug immunization regimen were found to have a significant ($p < 0.05$) increase in serum antibody levels to the IT-1 PS, whereas the nu/+ mice, similarly immunized, lacked detectable antibody (Table X). The antibody levels found in the nu/nu mice given the PS-drug immunization regimen were slightly higher than the antibody levels measured in the serum of nu/nu and nu/+ mice given an immunogenic 50-μg dose of the IT-1 PS. These data also showed that a response to the 50-μg dose of IT-1 PS could be generated in the absence of helper T cells.

Ability to induce protective immunity with IT-1 PS plus cyclophosphamide. Other investigators (11) demonstrated the ability of another immunopharmacologic agent, cyclophosphamide, to promote the development of CMI. To determine if the effects we observed with vinblastine could be duplicated by cyclophosphamide treatment, we immunized BALB/c mice with a wide range of doses of cyclophosphamide 48 hr before immunization with 1 μg IT-1 PS. We were unable to obtain protection to live organism challenge 7 days after the PS immunization at any of the doses of cyclophosphamide (Table XI). Thus, cyclophosphamide, over a wide dose range, was unable to duplicate the phenomenon observed with vinblastine, even though the conditions of cyclophosphamide administration were identical to those found to promote the development of CMI to cellular or protein antigens in other studies (11).

TABLE IX

Ability of spleen cell subpopulations to passively transfer immunity when taken from mice given 1 μg IT-1 high m.w. PS and 75 μg vinblastine

Cells[a] Treated on Plates Coated with:	SI[b] of Mitogens			No. Survivors Total recipients	P Value vs. Nonimmune Control
	PHA	Con A	LPS		
NMS	17.3	14.5	9.8	8/10	<0.001
Anti-Thy-1.2	1.4	1.7	8.2	2/10	0.24
Anti-Ig	11.4	12.2	1.9	9/10	<0.001
Nonimmune cells (control)	18.1	12.2	10.1	0/10	

[a] Originally 3×10^7 cells in 3 ml were placed on plates. After recovery cells resuspended in 1.5 ml HBSS and 0.5 ml given to recipient mice.

[b] SI, stimulation index, $\frac{\text{CPM, mitogen-stimulated cultures}}{\text{CPM, unstimulated cultures}}$.

TABLE X

Ability of 1 μg high m.w. PS plus 75 μg vinblastine to elicit protective immunity in nu/nu and nu/+ BALB/c mice

Immunized with	nu/nu Mice	Antibody Level (μg/ml)[a]	nu/+ Mice	Antibody Level (μg/ml)
50 μg PS	5[b]/5	19.8 ± 5.9	5[b]/5	21.2 ± 6.3
1 μg PS + 75 μg Vin	4/5	23.4 ± 8.1	4/5	<2.0
1 μg PS	1/5	<2.0	0/5	<2.0
75 μg Vin	0/5	<2.0	0/5	<2.0
PBS	0/5	<2.0	0/5	<2.0

[a] μg/ml antibody level determined in RABA assay.

[b] Represents number survivors over total challenged.

TABLE XI

Inability of cyclophosphamide plus 1 μg IT-1 high m.w. PS to induce immunity in BALB/c mice to live organism challenge

Dose (mg/mouse) Cyclophosphamide[a]	No. Survivors Total Immunized	P Value vs. PBS Control
0.0 (PBS control)	0/10	
0.4	2/10	0.24
1.0	0/10	1.0
2.0	0/10	1.0
3.0	2/10	0.24
4.0	0/10	1.0
5.0	0/10	1.0
PS only (1 μg)	0/10	1.0

[a] Cyclophosphamide administered i.v. via the tail vein in 0.2 ml ultrafiltered PBS 48 hr before a 1 μg i.p. dose of high m.w. PS in 0.5 ml ultrafiltered PBS.

DISCUSSION

The role of T cell-mediated immunity to infectious organisms is thought to be generally directed toward intracellular facultative parasites (4). Reports on the ability of activated macrophages to kill *Staphylococcus aureus* (12) and *Streptococcus pneumoniae* (13), as well as a role for T cell-dependent immunity in preventing abscess formation caused by *Bacteroides fragilis* (14), suggested extracellular bacterial pathogens can be killed by cellular immune mechanisms. Efforts to demonstrate the importance of T cells in protection from extracellular bacterial pathogens have been hampered by the efficiency of antibody-mediated effector mechanisms for protection against these organisms. In the studies reported here, we were able to induce specific protection against the extracellular bacterial pathogen, *P. aeruginosa*, IT-1, without stimulating antibody production. The protection observed could be adoptively transferred with immune spleen cells but not with serum from PS-vinblastine-immunized mice, and the effector cells were removed from immune spleen cell populations by antisera directed at the Thy-1.2 antigen present on T lymphocytes. Further information in nu/nu mice regarding the importance of T cells in the protection induced with IT-1 PS plus vinblastine was impossible to obtain because this immunization protocol allowed the development of antibody in these T cell-deficient mice. The reasons for this are unclear, but the data suggests that vinblastine relieves suppression in a non-T cell subpopulation of immunoregulatory cells. Nonetheless, it appears that a T cell-dependent immune effector mechanism can protect mice from live *P. aeruginosa* cell challenge following an appropriate immunization protocol. However, these data do not indicate what the specific T cell-dependent effector mechanisms are.

Because polysaccharide antigens are thought to be poor inducers of CMI, there was the possibility that a protein contaminant of the IT-1 PS preparation was responsible for the immunity elicited. Analyses of the composition of the IT-1 PS preparation does not support this possibility. Protein contamination of the IT-1 PS preparation is low (less than 1.0%) (5), indicating that any active protein contaminant would need to be effective at a dose of less than 0.01 μg. The serotype specificity of the protection would necessitate that a protein contaminant have a one to one association with the serotype determinant present on the PS and LPS of *P. aeruginosa*, a highly unlikely possibility. Furthermore, the major outer membrane proteins from strains of *P. aeruginosa* (which would be the most likely protein contaminants) are highly conserved among the seven Fisher strains used here (15). We have also recently tested (unpublished observation) the ability of perio

date to destroy the immunogenic activity of IT-1 PS, and found that periodate treated IT-1 PS is not effective in eliciting immunity in conjunction with vinblastine. Finally, the inability of cyclophosphamide, a drug known to enhance T cell immune responses to protein antigens (11), to reproduce the effects of vinblastine further argues against a critical role for a protein contaminant.

The role of vinblastine in augmenting the establishment of PS-induced T cell-mediated immunity is unclear. Cyclophosphamide-sensitive T suppressor cells have been shown to be responsible for interfering with the development of delayed-type hypersensitivity reactions to sheep red blood cells (11, 16). The lack of protection elicited by cyclophosphamide plus PS suggests a different cell, other than cyclophosphamide-sensitive T suppressors, is affected by vinblastine. Kappler and Hoffman (17) have shown that vinblastine can block the production of antibody by interfering with the division of B cells. Also, when vinblastine is administered at the proper time, it does not block the division of T cells that give rise to helper cell functions (17). It is possible that by interfering with B cell division and augmenting T cell responses vinblastine allows BALB/c mice to develop immunity to the normally nonimmunogenic 1-μg dose of IT-1 PS. Another possibility is that the vinblastine affects a macrophage regulatory cell. Gorczynski (18) identified distinct macrophage subpopulations that are involved in regulating antibody and CMI responses, and this latter cell may be affected by the vinblastine. Because vinblastine exerts its effects by interfering with RNA synthesis (19), it could potentially affect nondividing cells like macrophages by interfering with protein synthesis needed to suppress the development of immunity. Another possibility is that the vinblastine acts by directly stimulating the effector T cells, which are reactive to the IT-1 PS antigen. Although this explanation seems unlikely, it is not excluded by the present study.

These data indicate that the traditional concepts about immunity to extracellular bacterial pathogens need to be broadened. We demonstrated the ability of T cells, in the absence of antibody, to mediate protective immunity in mice to live organism challenge with *P. aeruginosa* IT-1. Our production of an i.p. infection in mice should not be considered to be a model of natural infection caused by *P. aeruginosa* in the compromised host. The role of CMI in protection against *P. aeruginosa* in natural infections is unclear. Reynolds *et al.* (20) demonstrated the release of macrophage inhibition factor (MIF) from respiratory cell cultures of rabbits immunized with LPS from *P. aeruginosa* IT-2 when the cell cultures were challenged with nontoxic amounts of LPS. Antigen stimulated lymphocytes from spleens of immunized rabbits likewise produced MIF when cultured with nonimmune macrophages as indicator cells. However, this ability to produce MIF was transient, and, in another study, Reynolds (21) showed that in spite of MIF production, *in vitro* cultured alveolar macrophages from immune animals were not capable of enhanced phagocytosis and intracellular killing of *P. aeruginosa*. Harvath *et al.* (22), using a granulocytopenic dog as a model for *P. aeruginosa* infections, showed that animals actively immunized with LPS had better survival rates than unimmunized animals. However, passive transfer of immune sera did not protect recipient animals despite high levels of circulating antibody, suggesting that in the actively immunized dogs a cell-mediated immune mechanism was needed for full protection. Antibody-mediated opsonophagocytosis of *P. aeruginosa* is thought to be the major immune protective mechanism against *P. aeruginosa* infection (23). However, the existence of clinical circumstances in which *P. aeruginosa* infections persist, despite high levels of specific antibody, as seen in cystic fibrosis patients (24), dictates a need for further study of the importance of T cell-mediated immunity to *P. aeruginosa*.

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Safety and Immunogenicity of High Molecular Weight Polysaccharide Vaccine from Immunotype 1 *Pseudomonas aeruginosa*

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ABSTRACT The safety and immunogenicity of a high molecular weight polysaccharide from immunotype 1 *Pseudomonas aeruginosa* were tested in a dose response fashion in adult volunteers. The vaccine lacked toxicity and pyrogenicity for experimental animals. Doses of 50, 75, 150, or 250 μg were given to groups of individuals as a single dose subcutaneous injection. Doses of 150 and 250 μg were associated with a significant rise in binding and opsonic antibody at 2 wk postimmunization. Titers remained unchanged for up to 6 mo. The vaccine was almost devoid of toxicity, eliciting no more than a slightly sore and tender arm at the site of injection. High molecular weight polysaccharide antigen appears to induce a good immune response following vaccination that is effective in mediating opsonophagocytic killing of live *P. aeruginosa* organisms.

INTRODUCTION

Infections caused by *Pseudomonas aeruginosa* have been particularly difficult to treat due to the organism's resistance to many antibiotics, the severity of the hosts' underlying condition that predisposes to *P. aeruginosa* infection, and the rapidity with which a septicemia can be fatal (1, 2). Immunotherapeutic modalities have been proposed as a potential means of increasing host resistance to this organism. Antibody directed towards cell surface lipopolysaccharide (LPS)[1] determinants has been shown to be effective in mediating opsonophagocytic killing of *P. aeruginosa* (3, 4). This antibody has been detected in the serum of patients convalescing from *P. aeruginosa* sepsis (5, 6), and survival of a *P. aeruginosa* sepsis episode has been associated with high levels of antibody to LPS in the acute phase serum (7). Attempts to induce antibody to LPS determinants in burn patients (8), cancer patients (9), and children with cystic fibrosis (10) have been hampered by the toxicity of LPS when used as a human vaccine. Nonetheless, these studies did suggest a drop in *P. aeruginosa* associated mortality following the use of an LPS vaccine. Recently, Jones et al. (11) documented the efficacy of a *P. aeruginosa* vaccine plus immunoglobulin in burn patients. Although the serologically active component of this vaccine has yet to be identified, the method of preparation (12) suggests it may be LPS.

A safe and immunogenic vaccine containing *P. aeruginosa* LPS serotype determinants would thus appear to be an ideal candidate for an immunotherapeutic agent to prevent *P. aeruginosa* sepsis. High molecular weight polysaccharide (PS) isolated from the supernate of *P. aeruginosa* cultures has been shown to be immunogenic in animals (13), to elicit protection to live organism challenge (14, 15) and to be nontoxic in mice and guinea pigs and nonpyrogenic in rabbits (14, 15). These PS antigens share serological specificity with the "O" specific side chain of LPS, yet differ from "O" side chains by their immunogenicity, biochemical constituents, monosaccharide composition, and molecular size (14, 15). Intact LPS contains the toxic lipid A component that is lacking in PS. Rabbit antisera to PS antigens contains antibody primarily directed at the LPS "O" side chain determinant, yet lacks anitbody to a second LPS-specific determinant present on the LPS molecule from all of the seven Fisher immunotypes of *P. aeruginosa*.[2] Thus animal

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[1] *Abbreviations used in this paper*: IT-1, immunotype 1; LPS, lipopolysaccharide.

[2] Pier, G. B., and D. M. Thomas. 1981. High molecular weight polysaccharide serotypes of *Pseudomonas aeruginosa*. Submitted for publication.

studies have shown that PS is capable of inducing an antibody response directed at LPS serotype determinants, yet lacks the toxicity associated with LPS vaccines. The present study was designed to assess the immunogenicity and safety of a prototype PS vaccine isolated from the immunotype 1 (IT-1) strain of *P. aeruginosa* in adult human volunteers, including an assessment of the functional nature of the antibody induced.

METHODS

Vaccine. High molecular weight PS antigen was extracted from a 30-liter culture of IT-1 *P. aeruginosa* grown in trypticase soy broth (Difco Laboratories, Detroit, Mich.) supplemented with 1% sodium acetate. After 48 h of growth, 300 g of hexadecyltrimethylammonium bromide was added and the precipitate and the organisms removed by centrifugation. The supernate was then concentrated to 800 ml on an Amicon TCE 5 ultrafiltration apparatus using PM 30 membranes (Amicon Corp., Danvers, Mass.). The crude PS-containing material was precipitated from the concentrate by the addition of 4 vol of 95% ethanol and recovered by centrifugation. This material was redissolved in phosphate-buffered saline (0.1 M phosphate, 0.15 M NaCl, pH 7.2) heated at 60°C for 1 h, and a one-tenth volume of 10% hexadecyltrimethylammonium bromide added to precipitate nucleic acids. After centrifugation, the supernate was recovered, crude PS precipitated by the addition of 4 vol of 95% ethanol, and the above procedure for removing nucleic acids repeated twice. Following this, the crude PS was dissolved in 1% acetic acid, the pH adjusted to 5.0 with glacial acetic acid, and the solution heated at 90°C for 18 h. This procedure cleaved the LPS into its lipid A and "O" side chain components for subsequent removal. After cooling, the lipid A precipitate was removed by centrifugation, the supernate extracted 10 times with chloroform then twice with 90% phenol, precipitated with 4 vol of 95% ethanol, redissolved in PBS, and applied to a Sephacyl S-300 column 2.6 × 100 cm in four separate runs. The serologically active material eluting between the void volume and the point where a 70,000-mol wt dextran marker begins to elute was collected, precipitated with alcohol, recovered, dialyzed, and lyophilized. This material was then weighed, dissolved to 1 mg/ml in pyrogen-free water with 1:30,000 merthiolate added and lyophilized as 1-mg aliquots in individual vials. Sterility of the material was ascertained in bulk before packaging and in 10% of the final packaged material in accordance with the Food and Drug Administration regulations (Title 21, Sect. 610.12). Prior to injection the vaccines were reconstituted with an appropriate amount of sterile saline for injection to give the desired dosage in 0.5 ml.

Chemical analyses. Analyses for nucleic acids, proteins, LPS, lipids, phosphate, carbohydrate, monosaccharide components, and water were performed as described (13).

Animal toxicity studies. The general safety test using guinea pigs (Title 21, Section 610.11) was done in two Hartley strain animals weighing 325 and 345 g. The animals were given 500 μg of PS in 5 ml saline, observed and weighed daily. The growth rate of 21 g mice was observed following intraperitoneal injection of 500 μg PS in 0.5 ml saline. Pyrogenicity was tested in three New Zealand White rabbits weighing between 2.04 and 2.50 kg following intravenous injection of 300 μg/kg body wt. Rectal temperatures were recorded prior to immunization and hourly for 3 h thereafter. Endotoxin contamination was tested for by the limulus lysate coagulation method (Sigma Chemical Co., St. Louis, Mo.). Two 12-kg rheuses monkeys were given four injections of 100 μg of PS subcutaneously at 3-d intervals and observed for local and systemic reactions for 72 h after each injection. Sera were collected before injection and weekly for 4 wk following the final injection.

Subjects. 42 normal healthy adult volunteers were asked to participate in this study. Signed informed consent was obtained, the volunteers randomly assigned to one of four groups receiving various doses of the vaccine, 20 ml of blood obtained by venipuncture, then a 0.5-ml subcutaneous injection of the vaccine given in the deltoid region of the arm. Subjects were interviewed at 24 and 48 h after the injection, symptoms noted, and temperatures recorded. Postimmunization sera were obtained at 14- and 28-d intervals following injection. For some subjects, serum was also obtained 6 mo postinjection.

Serologic methods. Serum antibody levels to the IT-1 PS were quantitated by means of a radioactive antigen binding assay using intrinsically labeled [^{14}C]PS prepared as previously described (13, 16). Sera were separated and stored at −20°C. Quantitation was performed as previously described for animal sera (16) except that five human sera were used to establish a standard curve. The correlation coefficient between percentage binding in the radioactive antigen binding assay vs. $\log_{10}$ microgram per milliliter of antibody was 0.901.

Opsonophagocytosis assays were performed by an adaptation of the methods of Baltimore et al. (17) and Young (4). Human peripheral blood leukocytes were purified on a dextran gradient, freed of erythrocytes, and suspended to 10^7 cells per milliliter. *P. aeruginosa* IT-1 was harvested in mid-log growth phase, washed once with minimal essential media (Microbiological Associates, Bethesda, Md.) and resuspended to 3×10^7 organisms/ml. The reaction mixture consisted of 100 μl of the serum or serum dilution to be tested, 100 μl of cells, 100 μl of organisms, and 100 μl of a 1:5 dilution of guinea pig complement. A 25-μl aliquot was removed from the tube at time 0, diluted in distilled water to lyse the leukocytes, then further diluted in saline and plated out on trypticase soy agar plates for bacterial enumeration. A similar aliquot was removed following 60 min of incubation at 37°C where tubes were continuously mixed, and organisms counted. The opsonic titer of the serum was expressed as the reciprocal of the serum dilution killing 90% or more of the initial inoculum. Controls for each experiment included mixtures of two of three components (cells, serum, and complement) plus organisms and media.

Statistical methods. Differences in the concentration of antibody in preimmunization and postimmunization sera were compared by a *t* test (18). Antibody titer rises of four-fold or greater in the opsonophagocytic assay were considered a positive response and analysis of responses between groups receiving different doses were compared by logistic regression (19).

RESULTS

Chemical analyses. The results of analyses for the various biochemical and monosaccharide constituents of the IT-1 high molecular weight PS are shown in

TABLE I
Chemical Analyses of the P. aeruginosa IT-1 Vaccine

	%
Component	
Carbohydrate (Total)	72.5
Lipid	<0.5
Phosphate	<0.5
Nucleic acid	0.8
Protein	0.9
Water	22.2
Monosaccharide constituents	
Arabinose	6.1
Rhamnose	3.4
Mannose	62.2
Galactose	19.8
Glucose	8.5

Table I. Consistent with previously published results (13) the antigen was composed principally of carbohydrate, with low levels of contaminating nucleic acids, protein, and LPS. A high level of mannose was also found along with the previously reported monosaccharides of arabinose, rhamnose, galactose, and glucose (13).

Animal studies. The results of animal tests and in vitro coagulation of the limilus amebocyte lysate for toxicity indicated that the PS vaccine passed these tests with no indication of toxicity. These tests were performed on final packed material rehydrated with sterile saline for injection. No significant rises in temperature (<0.5°F) were detected in rabbits given 300 μg/kg body wt. Further lack of biologically active endotoxins was seen in the limulus lysate assay, where it took 1,000 times more vaccine than control LPS to gel the lysate (Table II). General toxicity tests in guinea pigs, mice, and monkeys revealed normal weight gains following injection of up to 500 μg PS vaccine. The two monkeys given four 100-μg injections developed both binding antibody and titer rises of fourfold or greater by opsonophagocytosis.

Toxicity in human volunteers. Four different doses were given to volunteers: 7 persons received 50 μg, 5 persons received 75 μg, 12 persons received 150 μg, and 18 persons received 250 μg. Reactions to the PS vaccine were exceedingly mild, and no greater reaction than soreness and slight tenderness at the injection site were noted for any vaccinee at any dose, except for one person receiving 150 μg who was scratched by the needle under the injection site and developed a slightly red and tender area lasting for 48 h. No reaction lasted >48 h, and no erythema or induration was seen in any vaccinee other than as noted above. Slight soreness and tenderness at the injection site was seen in 1 of 7 (14.3%) persons given 50 μg, 1 of 5 (20%) persons given 75 μg, 4 of 12 (33.3%) persons given 150 μg, and 12 of 18 (66.7%) persons given 250 μg.

Antibody response. The antibody responses of subjects in each of the four dosage groups 2 and 4 wk postimmunization, as quantitated in the radioactive antigen binding assay, are shown in Table III. There was no significant difference between preimmunization and postimmunization concentrations of antibody in the group given 50 of 75 μg (*t* test). In the group given 150 μg a significant ($P = 0.004$) difference in the mean pre- and postimmunization antibody concentrations were noted. Similarly, at 250 μg a significant ($P = 0.002$) difference in antibody concentration was

TABLE II
Analyses for Endotoxin Contamination by Gelation of the Limulus Amebocyte Lysate

	Component		
Amount	IT-1 PS vaccine	IT-1 LPS	*Escherichia coli* LPS standard
ng			
0.1	−*	−	−
0.5	−	−	+
1.0	−	+	+
10.0	−	+	+
100.0	−	+	+
1,000.0	+	+	+

* + Indicates gelation of lysate; − indicates no gelation after 24 h.

TABLE III
Immunogenicity of PS Vaccine from IT-1 P. aeruginosa

	Antibody concentration geometric mean±SD (range), μg/ml		
	Weeks after immunization		
Dose	0	2	4
μg			
50	13.2±12.8 (3.9–40.0)	30.5±29.2 (4.0–88.4)	31.2±30.3 (4.0–86.8)
75	22.8±41.9 (4.1–97.8)	42.8±73.9 (3.8–174.3)	44.5±76.8 (3.8–175.2)
150	5.9±3.6 (1.7–14.9)	63.8±55.8 (6.1–147.4)	66.7±54.3 (6.1–155.8)
250	4.9±1.9 (3.6–10.2)	55.9±61.8 (3.7–250)	56.3±59.3 (3.7–232)

TABLE IV
Duration of Antibody Levels in Persons Receiving 150 μg of IT-1 P. aeruginosa PS Vaccine

Antibody concentration in μg/ml-geometric mean±SD (range)		
Time after immunization		
0	6 mo	Difference (post-pre)
5.9±3.6 (1.7–14.9)	32.7±29.3 (4.2–94.2)	27.3±28.8 (1.1–87.4)

noted. No significant difference was noted in the geometric mean antibody titer achieved at 2 wk when compared to 4 wk by a pooled *t* test, and no significant difference was noted between the geometric mean antibody concentration achieved in the sera of vaccinees receiving 150 μg (63.8 μg/ml) vs. that achieved at the 250-μg dosage (55.9 μg/ml). Serum antibody levels present at 6 mo following immunization with 150 μg are shown in Table IV. The decrease in individual antibody levels was not significant, indicating maintenance of antibody titers during this interval.

Functional properties of the induced antibodies were measured in an opsonophagocytosis test, and the titers determined for pre- and day 14 postimmunization sera are shown in Table V. 3 of 7 persons receiving 50 μg had a fourfold or greater increase in titer in this assay, while 2 of 5 persons receiving 75 μg, 9 of 12 persons receiving 150 μg, and 16 of 18 persons receiving 250 μg had these responses. In the total population, 32 persons had preimmunization titers of two or less, 8 had preimmunization titers of four through eight and 2 had preimmunization titers of more than eight. After immunization, 7 persons had titers of 2 or less, 13 had titers of 4–8 and 23 had titers of 1:16 or greater, up to 1:128. Logistic regression analyses of the dose response effect was performed on these data. Responses were designated 1 or 0 to indicate whether or not a fourfold or greater titer rise had occurred. The responses at 50 and 75 μg were treated as one category (low dose) for these statistical purposes. A highly significant difference ($P < 0.001$) was observed between the response seen in the group immunized with 150 and 250 μg, when compared to the response of the 50- and 75-μg group. The difference in response between 150- and 250-μg doses was marginally significant, ($P = 0.079$). This suggested a trend for the higher dose being slightly more efficacious in inducing a functional antibody response.

DISCUSSION

Disease due to *P. aeruginosa* infections is most often seen in immunocompromised or traumatized hosts. Susceptibility to infection has been thought to be correlated with granulocytopenia (2), though the underlying host condition was found to be a better indicator of the severity and outcome of *P. aeruginosa* sepsis (20). These immunocompromised patients, who are at risk for developing *P. aeruginosa* infections, are altered in their responses to immunological stimuli, and therefore may not respond to the PS antigen with humoral antibody, as was shown here for normal volunteers. Vaccination of granulocytopenic populations generally results in poor immune responses, but there are certain populations who are at high risk for *P. aeruginosa* infections that can be immunized prophylactically. Other populations, such as burn and trauma victims, may respond adequately to vaccination if given soon enough after injury. Immunosuppressed populations can potentially be immunized before or in between courses of therapy. Since the PS vaccine used here induced both binding and opsonic antibody, and has minimal toxicity in vaccinees, it offers the possibility to function as an effective immunotherapeutic agent for preventing *P. aeruginosa* sepsis.

TABLE V
Serum Titers in the Opsonophagocytosis Assay following Immunization with P. aeruginosa IT-1 PS Vaccine

Dose	Number immunized	Preimmunization titer			Postimmunization titer			No. persons with fourfold or greater rise
		≤2	4–8	>8	≤2	4–8	>8	
μg								
50	7	5*	2	0	3	2	2	3
75	5	4	1	0	3	1	1	2
150	12	9	1	2	1	5	6	9
250	18	14	4	0	0	5	13	16

* Represents number of vaccinees with this titer.

PS antigens are prepared by a method utilizing heat and acid to cleave the contaminating LPS into its lipid A, "O" side chain and core components for subsequent elimination. This somewhat harsh method was chosen because it was found to be the only method that removed all detectable intact LPS. Although low levels of LPS contamination in a vaccine may not be of any concern if the toxicity is within acceptable limits, animal studies of numerous *P. aeruginosa* vaccines have often indicated that contaminating LPS is the responsible agent for the immunogenicity and protective efficacy seen (14). The immunogenicity of this PS vaccine in humans, coupled with its almost total lack of toxicity, indicates that the acetic acid method for elimination of LPS is not only an effective procedure for reducing toxicity, but does not interfere with immunogenicity.

The magnitude of the human immune response to PS is particularly good when compared to the amount of antibody inducible in experimental animals (16). Humans by far had a greater degree of response than we have found for the most responsive laboratory animal, the C_3H mouse strain. The opsonic titers we obtained in our vaccinees was also close to that reported by Young and Armstrong (3) and Young (4) for patients recovering from *P. aeruginosa* sepsis or immunized with an LPS vaccine. The opsonophagocytosis test they used was very similar to the one used here, except that they multiplied their titers by a factor of 10 to translate the 0.1-ml amount of serum used in the reaction mixture to 1.0 ml. The data here report the dilution of a 0.1-ml amount of serum that elicited killing. Another slight difference was their use of ≥70% reduction in viable organisms as representing killing, while we used ≥90% levels. Taking this into account, the phagocytic titers of 12 persons recuperating from *P. aeruginosa* bacteremia ranged from 32 to 2,048 (our method), with 8 of these patients (66.7%) having titers of 32–128. 12 of 18 (66.7%) of the PS vaccinees receiving 250 μg had titers of 16–128. None of the immunized individuals had a titer of >128, whereas 4 of 12 of the infected patients did. Similarly in Young's (4) study of the opsonic titers of humans given monovalent preparations of *P. aeruginosa* LPS, opsonic titers of 256–2,048 were obtained. Although these titers are slightly higher than those obtained by PS vaccination, the dose of LPS used was quite high when compared to PS (25 μg/kg LPS vs. an average of 3.5 μg/kg PS) was given in five doses when compared with the single dose of PS, and was associated with local reactions not seen with PS immunization.

The influence of antibody levels of *P. aeruginosa* LPS serotype determinants in affecting the outcome of *P. aeruginosa* infection has been suggested by both vaccine studies (8, 9, 11) and also by a study of the influence on survival of acute phase antibody levels to LPS (7). Our studies in animals, (14, 15) indicate that PS induces a serotype specific immune response against the LPS "O" side chain determinant. Further study is required to assess whether the human immune response to PS vaccination also induces a response to LPS specific determinants. The data here do indicate that immunization with PS leads to an immune response in humans, that the antibody elicited can function in opsonizing live organisms, and that PS vaccination is associated with a minimal level of toxicity in vaccinees.

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